Functional analysis of *Agrobacterium tumefaciens* virulence protein VirD5

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Cover front: U2OS-TR cells transformed with plasmids encoding GFP-PCNA and mCherry-VirD5.

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Functional analysis of *Agrobacterium tumefaciens* virulence protein VirD5

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To my family!

Table of contents

Chapter 1

Introduction

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Cell division

The cell cycle is a complex process including the growth and the division of cells. Cell division takes place after two consecutive events, namely chromosome DNA duplication and segregation of the replicated chromosomes over two separate daughter cells. Cytologically, the cell cycle is divided in two stages: interphase and mitosis. Interphase is further divided into the G1, S and G2 phases. During the G1 phase, cells are preparing for DNA replication, whereafter DNA replication occurs in S phase, and in the following G2 phase, cells are preparing for mitosis. Mitosis is a continuous process, which is conventionally divided into five stages: prophase, prometaphase, metaphase, anaphase and telophase. In prophase, chromosomes start to condense into compact visible threads. Subsequently, each duplicated chromosome, consisting of a pair of sister chromatids intertwined with each other by cohesin proteins, is attached via a specific structure called the kinetochore to spindle microtubules emanating from the opposite poles. In metaphase the pairs of chromatids are aligned at the equator of the mitotic spindle. Once sister chromatids begin with separation, cells are said to be in anaphase, where chromosomes segregate to the opposite poles of the spindle. In telophase, chromosomes arrive at the opposite poles of the cell and decondense. During cytokinesis, the cell is physically separated into two units (Walczak, Cai, and Khodjakov, 2010; Zaidi *et al*., 2010; Zhu and Mao, 2015). Eukaryotic cells have evolved to tightly regulate mitosis by the so called spindle assembly checkpoint (SAC) to ensure that all

chromosomes are accurately segregated into daughter cells. A defect in this checkpoint allows mitosis to proceed with mistakes and thus leads to chromosome mis-segregation, chromosome instability, DNA damage and aneuploidy, which are common properties of cancer cells (Bakhoum and Compton, 2012; Crasta *et al*., 2012a; Giam and Rancati, 2015; Sotillo *et al*., 2007).

Centromere

The centromere (CEN) is the chromosomal locus that is required for the assembly of the kinetochore, through which the chromosome is attached to the spindle microtubules so as to divide the sister chromatids equally over the daughter cell during mitosis. Centromeric DNA is extremely diverse among eukaryotes, ranging from the simplest ~125bp centromeres present on each of the chromosomes of *Saccharomyces cerevisiae* to the highly repetitive α-satellite regions of vertebrates (Verdaasdonk and Bloom, 2011). The yeast CEN contains three distinct elements, CDEI, CDEII and CDEIII (Carbon and Clarke, 1984). CDEI binds to the non-essential Cbf1 protein (Cai and Davis, 1989), CDEII embraces an A+T-rich segment (~80bp), which is bound by specialized nucleosomes with the highly conserved protein Cse4/CENP-A, a histone H3 variant that replaces canonical H3 at centromeres. (Meluh *et al*., 1998). CDEIII is bound by the CBF3 complex, which contains Ctf13, Ndc10, Cep3 and Skp1 (Cho and Harrison, 2012; Cole, Howard, and Clark, 2011). Although sequences of centromeres from different organisms are very different, they are all specified by incorporation of a special histone H3 variant, forming a specific nucleosome platform for the assembly of the kinetochore (Sullivan, Hechenberger, and Masri, 1994; Talbert *et al*., 2002).

Protein composition of the kinetochore

The kinetochore assembles on the centromere and interacts with spindle microtubules to establish bipolar attachment of paired sister chromatids during mitosis. The kinetochore is a large complex of proteins consisting of an inner kinetochore that interacts directly with the centromeric DNA, and an outer kinetochore that connects the kinetochore to the spindle microtubules (**Figure 1**) (Burrack and Berman, 2012; Cheeseman and Desai, 2008; Cho and Harrison, 2012). The centromeric nucleosomes, characterized by the presence of the histone H3 variant Cse4 (CENP-A in higher eukaryotes), directly interact with the inner kinetochore protein complexes, which is in higher eukaryotes called the constitutive centromere associated network (CCAN) (Hori *et al*., 2008; Lampert and Westermann, 2011). CCAN includes four other proteins with a histone fold (Cnn1/CENP-T, Mhf1/CENP-S, Mhf2/CENP-X and Wip1/CENP-W). Also proteins of the Cbf3 complex interact directly with the centromeric nucleosomes. This complex in turn recruits Mif2/CENP-C, which in its turn helps recruit the Ctf19 complex via interaction with Iml3/CENP-L (Cheeseman *et al*., 2006; Sullivan *et al*., 1994). There are several direct links between inner and outer kinetochore proteins. The Cnn1/CENP-T protein directly binds to Ndc80 and the Mif2/CENP-C protein interacts with Nnf1, a subunit of the Mis12 complex (Wan *et al*., 2009). An important regulatory complex, which binds transiently from prophase to metaphase at the centromeres, is the Chromosomal Passenger Complex (CPC), which embraces the regulatory Aurora kinase Ipl1, Sli15/INCENP, Nbl1/Borealin and Bir1/Survivin

(Carmena *et al*., 2012). The outer kinetochore is made up of the KMN complex, which consists of the KNL1 complex (Spc105 complex in yeast), Mis12 complex (Mtw1 complex in yeast) and Ndc80 complex (Lampert and Westermann, 2011). A ten-protein Dam1 complex in yeast has been shown to form a stable ring structure encircling the microtubules. Through interactions of the Dam1 complex with the Ndc80 complex, a bridge can be formed between the kinetochore and spindle microtubules (Miranda *et al*., 2005; Westermann *et al*., 2006). However, the Dam1 complex is only present in fungi, and is replaced by the unrelated Ska1 complex in mammalian cells, which functions however similarly as the yeast Dam1 complex in connecting the spindle microtubules to the Ndc80 complex (Schmidt *et al*., 2012).

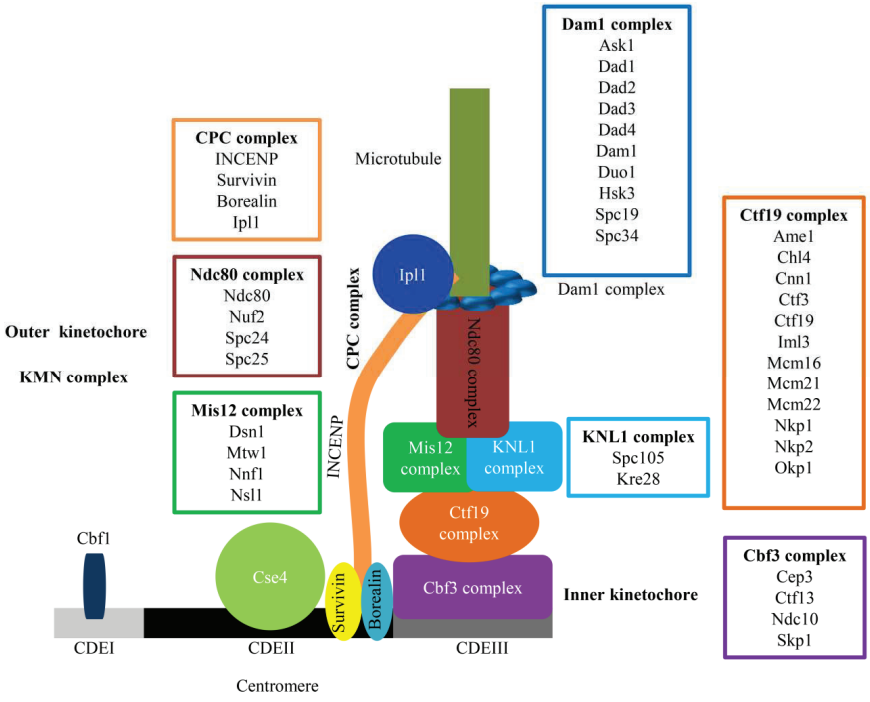

Figure 1. Yeast centromere-kinetochore.

Kinetochore-microtubule attachment

Accurate distribution of chromosomes relies on the attachment of the paired sister chromatids to spindle microtubules emanating from opposite poles. Erroneous bindings including syntelic attachment (sister kinetochores attach to the spindle microtubules from the same pole) and merotelic attachment (a single kinetochore binds to spindle microtubules from both poles) must be corrected before cytokinesis (**Figure 2**), as otherwise these improper bindings may cause aneuploidy, the generation of cells with an abnormal number of chromosomes (Godek, Kabeche, and Compton, 2014; Gregan *et al*., 2011). Correcting mechanisms sense the tension caused by kinetochore attachment to the spindle microtubules, while sister chromatids are

still tethered by cohesin and can discriminate improper bindings from correct ones (Sacristan and Kops, 2015; Saurin *et al*., 2011; Tanaka *et al*., 2000). Tension across sister kinetochores is generated in bipolar attachments because of the pulling forces from microtubules emanating from the opposite poles on sister kinetochores. When there is a lack of tension across a pair of sister kinetochores, an Aurora kinase (Ipl1 in yeast) promotes the destabilization of kinetochore-microtubule attachments by phosphorylating proteins involved in this attachment. The unbound kinetochore may then find another microtubule to bind to (Buvelot *et al*., 2003; Tanaka, 2005).

The Dam1 and Ndc80 proteins, key components of kinetochore-microtubule attachment, are among the substrates of the Aurora B/Ipl1 kinase. The phosphorylated proteins cause kinetochore detachment from microtubules. Ndc80, known as HEC1 in human cells has an extensive N-terminal tail, which contains seven Aurora B/Ipl1 kinase phosphorylation sites (DeLuca, Lens, and DeLuca, 2011; Wei, Al-Bassam, and Harrison, 2007). Phosphorylation of Ndc80 at its N-terminal tail by Aurora B strongly reduces its attachment to microtubules (Alushin *et al*., 2012). Cells expressing Ndc80/HEC1 mutants lacking these phosphorylation sites display chromosome alignment defects (DeLuca *et al*., 2011). Ska1 harbors a C-terminal microtubule-binding domain with four Aurora B kinase phosphorylation sites (T157, S185, T205 and S242). Mutation of these phosphorylation sites to aspartate (phosphorylation mimic)

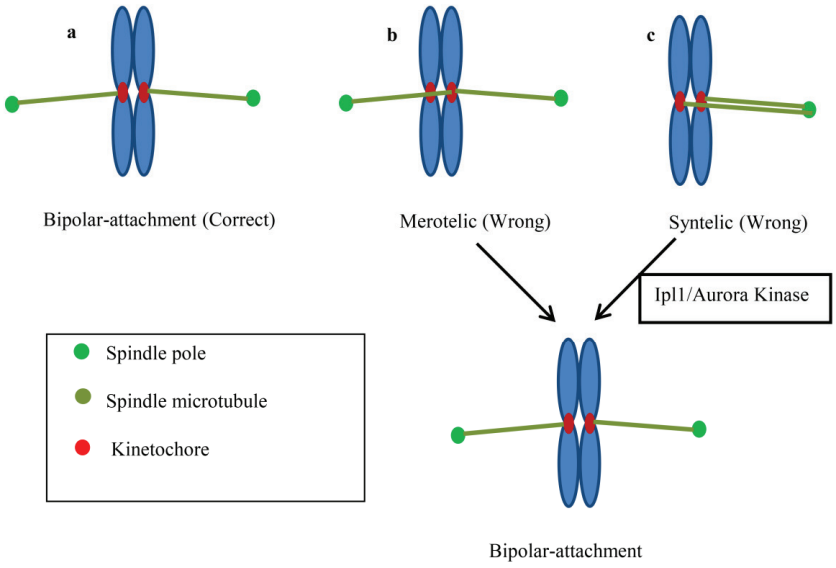

Figure 2. Modes of kinetochore-microtubule attachment. (a) Stable bipolar-attachment. (b) Merotelic attachment: single kinetochore attached to spindle microtubules from both poles. (c) Syntelic attachment: both sister kinetochores attached to spindle microtubules from the same pole. (d) Bipolar-attachment after detachment by Ipl1/Aurora kinase.

causes a mitotic delay and reduced kinetochore binding (Chan *et al*., 2012; Schmidt *et al*., 2012). Similarly, the yeast Dam1 protein contains several Ipl1 phosphorylation sites (S20, S257 and S265) (Westermann *et al*., 2006), mutation of which to aspartic acid (phosphorylation mimic) causes chromosome lagging in the middle of the spindle, resembling the consequences of detached kinetochores (Cheeseman et *al*., 2002).

Aurora kinase

Aurora kinases belong to a family of highly conserved serine/threonine protein kinases that play essential roles in many key processes during mitotic cell division. They mainly consist of two functional domains, a conserved C-terminal catalytic domain required for the kinase activity, and an N-terminal variant regulatory region that is responsible for interactions with distinct substrates for the subsequent phosphorylation by the C-terminal catalytic domain (Fu *et al*., 2007).

In budding yeast, there is one unique Aurora kinase called Ipl1 that was originally identified as a stimulator of ploidy (Chan and Botstein, 1993). Ipl1 is present at the kinetochores from G1 to metaphase, moves to the spindle after metaphase, and stays at the spindle midzone during late anaphase (Buvelot *et al*., 2003). The protein promotes chromosome bi-orientation by inducing the detachment of erroneous kinetochore-microtubule attachments and is also involved in later stages of mitosis up to cytokinesis (Norden *et al*., 2006; Tanaka *et al*., 2002). Several kinetochore components have been identified as the targets of Ipl1 kinase, such as the inner kinetochore protein Ndc10, the microtubule embracing protein Dam1 and the outer kinetochore protein Ndc80 (Akiyoshi *et al*., 2009; Biggins *et al*., 1999; Keating *et al*., 2009). Phosphorylation of Dam1 and Ndc80 by Ipl1 leads to kinetochore-microtubule detachment, thereby at the same time activating the SAC. This regulatory process thus plays essential roles in faithful chromosome segregation by allowing the correction of erroneous attachments between kinetochore and microtubule (Cheeseman *et al*., 2002; Pinsky *et al*., 2006).

Unlike budding yeast, there are three Aurora kinases in mammalian cells, Aurora A, Aurora B and Aurora C, each of which has distinct locations and functions during cell division. Aurora A accumulates at centrosomes and has essential roles in centrosome maturation, spindle assembly and correction of erroneous kinetochore-microtubule attachment (Dutertre, Descamps, and Prigent, 2002; Ye *et al*., 2015). Ectopic expression of Aurora A has been shown to cause centrosome amplification, chromosome instability and aneuploidy, consequently triggering tumorigenesis (Maia, van Heesbeen, and Medema, 2014; Zhou *et al*., 1998). Aurora B kinase assembles as part of the CPC complex with three other components: INCENP, Survivin and Borealin (Carmena *et al*., 2012). It is present at the centromeres from prometaphase to metaphase and then transfers to the midzone and persists at the midbody until cytokinesis is completed (Carmena *et al*., 2012). These dynamic changes in its sublocation during the cell cycle ensure the effective phosphorylation of substrates involved in chromosome condensation, SAC, kinetochore-microtubule attachment and cytokinesis. The central role of Aurora B kinase is to control accurate chromosome segregation by destabilizing wrong attachments between kinetochore and microtubule by phosphorylating core substrates (Ndc80, Ska1) that are involved in the kinetochore-microtubule attachment (Chan *et al*., 2012; Ciferri *et al*., 2008). Due to its essential role

during mitosis, ectopic expression of Aurora B kinase such as in cancer cells leads to chromosome instability, chromosome mis-segregation, aneuploidy and micronuclei (Lin *et al*., 2010; Takeshita *et al*., 2013). Aurora C, seems to have a similar dynamic sublocation in the cells during mitosis as Aurora B, suggesting overlapping functions with Aurora B kinase (Sasai *et al*., 2004). However, recent research showed that Aurora C has also distinct functions from Aurora B in chromosome alignment and kinetochore-microtubule attachments in the metaphase of meiosis I (Balboula and Schindler, 2014).

Like human cells, plants also harbor three distinct Aurora kinases called Aurora1, Aurora2 and Aurora3. Aurora1 and Aurora2 can be classed into the same group due to their similar dynamic locations during cell division (nuclear membrane in interphase, spindle from prophase to metaphase, and midzone during anaphase), whereas Aurora3 exhibits a distinct location, and thus belongs to another group (Kawabe *et al*., 2005). Ectopic expression of Aurora kinases in plant cells leads to chromosome mis-segregation and polyploidy and aneuploidy (Demidov *et al*., 2014).

Chromosome segregation

During DNA replication, the duplicated sister chromatids are tethered together by cohesin rings that are made up of two structural maintenance of chromosome (SMC) proteins (**Figure 3**), SMC1 and SMC3, and two other subunits, the kleisin subunit (Scc1) and Scc3 (in mammalian cells in two forms called SA1 or SA2) (Haering *et al*., 2008; Peters, Tedeschi, and Schmitz, 2008). Once all sister kinetochores have properly been attached to spindle microtubules emanating from the opposite poles, the anaphase-promoting complex/cyclosome in conjunction with its cofactor Cdc20 (APC/C^{Cdc20}) is no longer inhibited. The active APC/C^{Cdc20} targets securin for degradation so that separase becomes active, which can cleave the kleisin subunit of cohesin, opening the cohesin rings so that sister chromatids can be separated into daughter cells in anaphase. In contrast to yeast

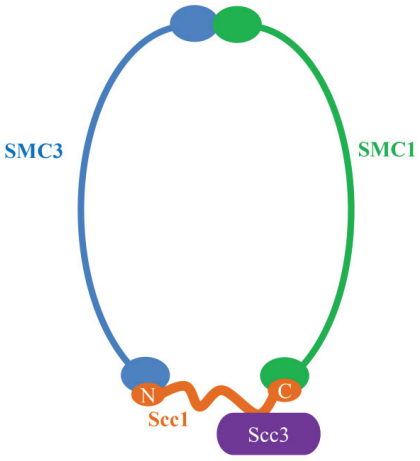

Figure 3. Overview of the structure of cohesin ring.

(Marston, 2014), in vertebrate cells, cohesin release occurs in two steps. Cohesin disappears from chromosome arms without Scc1 cleavage after phosphorylation of the SA2 cohesin subunit by Aurora B and Plk1 kinases before the onset of anaphase, whereas the centromere-bound cohesin is released by separase once cells enter anaphase (Hauf *et al*., 2005; Losada, Hirano, and Hirano, 2002; Sumara *et al*., 2002). How phosphorylation of SA2 mediates the dissociation of cohesin from chromosome arms remains elusive. The protection of cohesin at the centromeres is mediated by a highly conserved shugoshin (Sgo1) protein (Watanabe and Kitajima, 2005), which recruits the phosphatase 2A (PP2A) complex to centromeres via a direct interaction. The PP2A complex dephosphorylates the SA2 subunit, consequently protecting cohesin against dissociation at centromeres (Bollen, Gerlich, and Lesage, 2009). The Sgo1 protein is conserved in yeast where it is known to recruit condensin, which is involved in chromosome condensation to the centromeric region through interaction with the PP2A subunit Rts1, and to help maintain the Ipl1/Aurora B kinase on centromeres, which is important for subsequent correction of erroneous kinetochore-microtubule attachments (Peplowska, Wallek, and Storchova, 2014).

Chromosome instability, aneuploidy, micronuclei and tumor formation

Faithful chromosome segregation during mitosis is a prerequisite for maintaining the genetic material; any errors in the segregation may lead to chromosome instability (CIN), ultimately triggering aneuploidy and tumorigenesis. Several different mechanisms can drive CIN, including centrosome abnormality, improper kinetochore-microtubule attachment, a defective or hyperactive SAC, and premature release of cohesin (Gordon, Resio, and Pellman, 2012; Thompson, Bakhoum, and Compton, 2010).

The centrosome functions as the microtubule-organizing center (MTOC) to nucleate the spindle microtubules from both poles of the cell, forming a stable bipolar spindle. An abnormal number of centrosomes has been reported in many cancer cells, which also showed CIN and aneuploidy (Giehl *et al*., 2005; Vitre and Cleveland, 2012). Correct kinetochore-microtubule attachment supports the accurate segregation of chromosomes. Erroneous attachments including merotelic and syntelic attachments increase the frequency of chromosome mis-segregation if they are not corrected by the key regulatory proteins like Aurora kinases. Thus, mutation of these regulatory kinases may cause chromosome instability, whereas continuous detachment by overexpression of Aurora kinases also triggers chromosome mis-segregation, and may lead to tumorigenesis (Buvelot *et al*., 2003; Godek *et al*., 2014; Hégarat *et al*., 2011; Muñoz-barrera and Monje-casas, 2014; Hégarat *et al*., 2011).

Cells have developed a surveillance mechanism called SAC to ensure proper spindle-kinetochore attachments. The SAC causes a delay in the onset of anaphase until all paired sister chromatids have been attached by spindle microtubules emanating from two poles (Musacchio and Salmon, 2007). Thus, it is not surprising that mutations in SAC genes like BUB1 and MAD2 have been shown to induce tumorigenesis (Hanks *et al*., 2004; Michel *et al*., 2004). Overexpression of SAC genes may also cause aneuploidy and cancer (Ricke, Jeganathan, and van Deursen, 2011; Sotillo *et al*., 2007), since this persistent SAC may lead to tetraploid cells which are prone to be mis-segregated (Fujiwara *et al*., 2005). Cohesin rings tether sister chromatids together prior to the onset of anaphase. Defects in sister chromatid

cohesion also can lead to chromosome instability and tumorigenesis (Haering *et al*., 2008; Sajesh, Lichtensztejn, and McManus, 2013).

Micronuclei, a hallmark of the cells in solid tumors, are generated from lagging chromosomes or chromosome bridges after mitosis. Their presence is considered to be an indicator of genotoxicity and chromosome instability. Many factors contribute to the formation of micronuclei including mis-repair of DNA damage, erroneous chromosome attachments and chromosome fragmentation (Crasta *et al*., 2012b; Hayashi and Karlseder, 2013).

Tumor formation on plants by *Agrobacterium tumefaciens*

The Gram-negative soil bacterium *Agrobacterium tumefaciens*, is capable of infecting a large number of dicotyledonous plants, causing crown gall disease. The galls that are formed on plants represent tumors consisting of transformed cells that no longer require external plant growth regulators for division. Crown gall cells contain a small segment of DNA, the T-DNA, that originates from the tumor-inducing (Ti) plasmid of the bacterium. Although of bacterial origin the T-DNA contains genes that are expressed in plant cells. The finding that these T-DNA genes encode enzymes that catalyze the production of an auxin and a cytokinin, the classical plant growth regulators that drive cell division in plant cells, explained why T-DNA containing plant cells behave as tumor cells and why galls/tumors are formed on plants by the infection (Bochum, 1985; Zambryski, Tempe, and Schell, 1989).

The Ti plasmid has a size of about 200,000 bp and the T-DNA forms only a small part of it. The genes involved in the transfer of the T-DNA into plant cells are not encoded by the T-DNA itself, but by an adjacent part of the Ti plasmid, the Virulence region, embracing 20-30 *vir* genes. Phenolic compounds like acetosyringone that are released from plant wounds trigger the expression of these virulence genes (Stachel and Zambryski, 1986). As a consequence, a T-pilus is expressed on the surface of the bacterium. This T-pilus represents the position of a Type IV Secretion System (TFSS), which is built from the eleven different VirB proteins and the coupling protein VirD4 (Christie, 2004). At the same time, T-strands, single-stranded copies of the T-DNA are produced in the bacterium by the action of the VirD2 relaxase in cooperation with VirD1. The VirD2 protein remains covalently bound at the 5' end of the T-strand, forming a T-DNA-protein complex (T-complex), which is subsequently transferred into host cells via the TFSS. Once inside the nucleus of host cells, the T-strand can integrate into the host genome at DNA break sites (Christie, 2004; Ghai and Das, 1989; Pansegrau *et al*., 1993; Scheiffele, Pansegrau, and Lanka, 1995).

The natural property of *Agrobacterium tumefaciens* to transfer DNA into plant cells has led to the development of a variety of applications in plant biotechnology. In the Ti plasmid the T-DNA is embraced by two imperfect 24 bp direct repeats, called the Left border and the Right border, which are recognized and nicked by the VirD2 relaxase. The genes naturally present between the two repeats are not involved in DNA delivery and thus can be replaced by any other interesting genes. Vector systems based on this principle such as the binary vector system, are used now routinely to generate transgenic plants in the laboratory and for the genetic modification of crops. After it was discovered that *Agrobacterium* can also be used for the transformation of yeast and fungal cells, the bacterium has also become an important gene vector for various fungi and mushrooms (Bundock *et al*., 1995; Bundock and

Hooykaas, 1996; de Groot *et al*., 1998). The T-DNA is integrated in the plant genome preferentially by a pathway of non-homologous recombination (Offringa *et al*., 1990), and genomic double-strand breaks seem a preferred point of entry (Salomon and Puchta, 1998). Although the precise mechanism of T-DNA integration in plants remains elusive, in the model yeast *Saccharomyces cerevisiae* T-DNA integration occurs at DNA breaks either by non-homologous end joining (NHEJ) or by homologous recombination (HR). Inactivation of both of these pathways by mutation of the key genes for Ku70 (NHEJ) and for RAD52 (HR) prevented T-DNA integration all together (Van Attikum, Bundock, and Hooykaas, 2001; Van Attikum and Hooykaas, 2003). The results indicate that host enzymes are largely or entirely responsible for T-DNA integration and therefore integration occurs preferably by non-homologous recombination in plant cells, but by homologous recombination in yeast cells (Bundock *et al*., 1995; Bundock and Hooykaas, 1996).

Translocation of virulence proteins

Bacteria have evolved several systems to secrete proteins across the cellular membranes and even into host cells. These distinct secretory systems have been named Type I to IX secretion systems and each has a set of characteristic components. In particular, the Type III, IV, and VI systems are known to be involved in the interactions with target cells and to be capable of introducing virulence (effector) proteins into target cells. The most widely-studied Type III Secretion System (TTSS) is used by bacterial pathogens to inject their virulence proteins into eukaryotic host cells (Coburn, Sekirov, and Finlay, 2007). The Type IV Secretion System (TFSS) is related to the bacterial conjugation system, and is unique in that it can translocate both proteins and DNA molecules. It is used by certain bacterial pathogens for the delivery of virulence proteins into host cells and by the plant pathogen *Agrobacterium* to introduce both T-DNA and virulence proteins into plant cells (Christie and Vogel, 2000). The Type VI Secretion System (T6SS) is a bacteriophage-like device involved in transportation of a variety of toxic virulence proteins to kill or inhibit neighboring bacteria and is very important in interbacterial competition. Some bacteria seem to use a T6SS for delivery of virulence proteins into eukaryotic cells (Basler *et al*., 2012; Pukatzki *et al*., 2007). The effector proteins translocated by the various T3SS, T4SS and T6SS differ in their biological functions, but they all act to facilitate competition or promote infection by diminishing the host defense response or by modulating host functions to allow entry or maintenance of the pathogen (**Table 1**). Enzymatic functions of effectors include (a) nuclease activity, such as the CdiA-CT protein from *Escherichia coli* strain 536 (UPEC536) that has been shown to be an exported effector protein that cleaves the anti-codon loops of tRNA in targeting cells (Diner *et al*., 2012), (b) protein and nucleic acid modification, like the *Corynebacterium diphtheriae* diphtheria toxin (DT) that ADP-ribosylates eukaryotic elongation factor-2 (eEF2) to inhibit protein synthesis (Bennett and Eisenberg, 1994; Mateyak and Kinzy, 2013), (c) protein ubiquitination leading to degradation, for example, the *Shigella* IpaH9.8 effector that has a conserved C-terminal motif with E3 ubiquitin ligase activity (Zhu *et al*., 2008), (d) cell membrane leakage, such as the *Pseudomonas aeruginosa* effector ExoU, which is a potent phospholipase exported by the TTSS pilus into the membrane of mammalian cells (Rabin *et al*., 2006; Schmalzer, Benson, and Frank, 2010), and (e) protein phosphorylation, like the *Escherichia coli* O157:H7 type III effector EspG, which has been shown to stimulate three

eukaryotic p21-activated kinases (PAKs), consequently inhibiting host trafficking events (Selyunin *et al*., 2011). Further examples can be seen in **Table 1**.

Table 1. Bacterial effector proteins and their targets.

Bacterial species	Effector	Target	Mechanism of action
Bacills subtilis	WapA	tRNA	Nuclease
Dickeya dadantii 3937	RhsA ,RhsB	DNA	Nuclease
Escherichia coli strain 536	CdiA-CT	t-RNA	Cleavage at anti-codon loops
Agrobacterium tumefaciens	Tde	DNA	Nuclease
Pseudomonas aeruginosa	ExoS	Cdc42, Rac1,RhoA	ADP-ribosylation
Pseudomonas aeruginosa	ExoT	Crk	ADP-ribosylation
Corynebacterium diphtheriae	DT	eEF2	ADP-ribosylation
Salmonella enterica	SpvB	Actin	ADP-ribosylation
Pseudomonas syringae	HopU1	GRP7	ADP-ribosylation
Photorhabdus luminescens	TccC3	Actin	ADP-ribosylation
Photorhabdus luminescens	TccC5	Rho GTPase	ADP-ribosylation
Legionella pneumophila	DrrA	Rab1	AMPyaltion
Legionella pneumophila	AnkX	Small GTPase	AMPyaltion
Vibrio parahaemolyticus	VopS	Rho, Rac,Cdc42	AMPyaltion
Histophilus somni	IbpA	Rho GTPases	AMPyaltion
Xanthomonas citri	X-Tfe^{XAC2609}	PG	Cell membrane leakage
Pseudomonas aeruginosa	ExoU	Lipid	Cell membrane leakage
Pseudomonas aeruginosa	PldA,PldB	PE	Cell membrane leakage
Vibrio cholera	VgrG-3	PG	Cell membrane leakage
Salmonella SPI-2	SseJ	Lipid	Cell membrane leakage
Agrobacterium tumefaciens	VirF	VIP1	Ubiquitination
Shigella flexneri	IpaH9.8	Ste7	Ubiquitination
Salmonella enterica	Slrp	thioredoxin	Ubiquitination
Pseudomonas syringae	AvrPtoB	Fen	Ubiquitination
Legionella pneumophila	Lubx	SidH, CIK1	Ubiquitination
Escherichia coliO157:H7	EspG	PAKs	Enhance kinase activity
Shigella flexneri	OspE1,OspE2	PDLIM7, PKC	Enhance kinase activity
Yersinia pseudotuberculosis	YopJ	MAPK-ERK, JNK,	Inhibit kinases activity

PG: peptidoglycan; PE: phosphatidylethanolamine

Agrobacterium delivers the virulence proteins VirE2, VirE3, VirD5 and VirF into host cells independently of the T-complex via its VirB TFSS apparatus (**Figure 4**). These virulence proteins share a highly conserved positively-charged C-terminus essential for protein delivery by the TFSS into host cells (Vergunst *et al*., 2000; Vergunst *et al*., 2005). The VirE2 protein is the most important of these as tumor formation is strongly reduced in its absence. The VirE2 protein is a protein that binds cooperatively to single-stranded DNA *in vitro* and therefore is supposed to coat the T-strand (ssDNA) in host cells to protect it from nuclease attack (Rossi, Hohn, and Tinland, 1996; Gelvin, 1998; Grange *et al*., 2008). It interacts in plant cells with VIP1 (Tzfira, Vaidya, and Citovsky, 2001), a transcription factor containing a bZIP motif involved in the defense response, possibly forming a compact ternary complex with VIP1 and the T-complex, the T-strand with VirD2 at its 5'end. During infection VIP1 is phosphorylated and then is targeted to the nucleus, where it mediates transcription of defense genes. By binding to the T-complex with VirE2, it may enhance the uptake of the T-complex into the nucleus of plant cells. The VirE3 protein has itself strong nuclear localization signals and thus is delivered efficiently into the nucleus of host cells, via the interaction with plant importins α, which is involved in nuclear protein import (García-Rodríguez, Schrammeijer, and Hooykaas, 2006; Lacroix *et al*., 2005). It has been reported that it can interact with VirE2 in both yeast and plant cells, and may mimic the function of VIP1 by enhancing the transport of VirE2 and the T-complex into the nucleus. It has also been shown that VirE3 interacts with pCsn5, a subunit of the COP9 signalosome, and pBrp, a

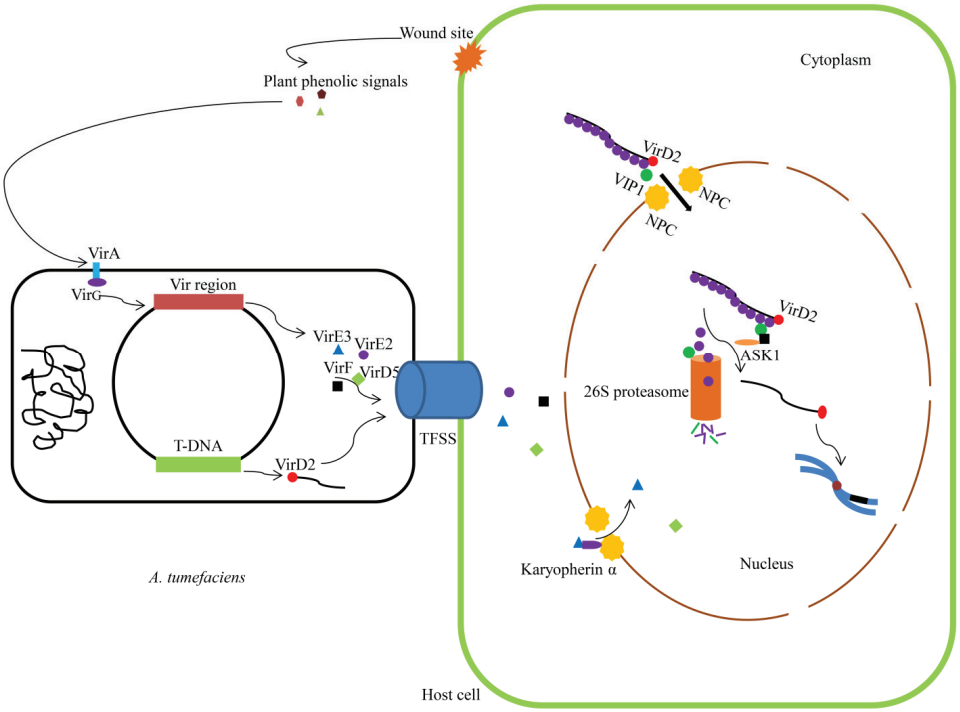

Figure 4. Schematic overview of the main process of *Agrobacterium tumefaciens*.

plant-specific TFIIB-related transcription factor, suggesting that VirE3 functions as a potential transcriptional activator in host cells (García-Rodríguez, Schrammeijer, and Hooykaas, 2006). A recent paper from our lab has shown that VirE3 together with pBrp stimulates the expression of several genes including that encoding VBF, a plant F-box protein that was reported to be able to replace the *Agrobacterium* VirF protein in infection (Niu *et al*., 2015; Zaltsman *et al*., 2010). VirF, the first described prokaryotic F-box protein (Schrammeijer *et al*., 2001), has been reported to bind to VIP1 and subsequently trigger the proteasomal degradation of both VIP1 and VirE2, if that is bound to it. It is thus hypothesized that the T-complex may be uncoated facilitating integration of the T-DNA into the genome of the host cells (Tzfira, Vaidya, and Citovsky, 2004). VirD5 is another effector protein that is transferred from the bacterium to host cells. It has been reported that VirD5 stabilizes the VirF protein in host cells by interaction with each other (Magori and Citovsky, 2011). Another group recently published that VirD5 plays dual roles in regulating host gene expression by a transcription activator domain at its N-terminus and in protecting VIP1 and VirE2 against degradation by the host 26S proteasome apparatus via competing with the host F-box protein VBF for binding to VIP1 (Wang *et al*., 2014). In this thesis, I have studied the function of VirD5 in yeast, plant and mammalian cells and found that it is targeted to the nucleus, where it affects the activity of the essential mitosis regulatory Aurora kinases that are essential for cell division and correct chromosome segregation. The action of VirD5 caused chromosome mis-segregation, micronucleus formation and aneuploidy, all hallmarks of tumor cells.

Outline of this thesis

Agrobacterium tumefaciens transfers a segment of DNA (T-DNA) from its tumor-inducing (Ti) plasmid to the nucleus of plant cells, where it is integrated into the nuclear genome and expressed. The expression leads to the uncontrolled growth of the transformed cells and ultimately causes tumor formation, the symptom of crown gall disease. Several virulence proteins facilitating transformation are delivered into host cells independently of the T-DNA. This study mainly focuses on the molecular functions of one of these translocated virulence proteins called VirD5.

Chapter 2 describes the highly conserved toxic activity of VirD5 from different *Agrobacterium* strains in target cells. The toxicity could be suppressed in yeast by nine yeast deletion mutants. One of these suppressive mutants lacks the SPT4 gene, which is involved in transcription elongation and kinetochore assembly. The Spt4 protein had a similar sublocation as VirD5 in yeast at the centromeres/kinetochores and was found to physically interact with VirD5. In the absence of Spt4, the localization of VirD5 at the centromeres/kinetochores and its toxicity were lost, suggesting that Spt4 facilitates the accumulation of the VirD5 protein at the centromeres/kinetochores foci and thereby facilitating its toxicity. Ectopic expression of VirD5 in yeast cells led to chromosome mis-segregation and massive DNA breaks.

Chapter 3 shows that VirD5 interacted with two other kinetochore-associated proteins, viz Dam1, an outer kinetochore protein encircling the spindle microtubules and Ipl1, the yeast Aurora kinase involved in restoring erroneous kinetochore-microtubule attachments during mitosis. Targeting the Ipl1 kinase by VirD5 stimulated its kinase activity on key

substrates involved in kinetochore-microtubule binding and consequently triggered cell cycle arrest in M phase. The presence of VirD5 caused chromosome mis-segregation, DNA damage and aneuploidy, which are all hallmarks of cancer cells.

Chapter 4 describes the toxic activity of VirD5 in *Arabidopsis thaliana*. It was found that VirD5 could also interact with the three plant Aurora kinases and might as a consequence affect their kinase activities. Transgenic plants containing *virD5* under the control of the tamoxifen inducible promoter showed defects of plant root meristem development even at a low dosage of VirD5 and chromosome mis-segregation, and in the end inhibited plant growth. Expression of VirD5 from a strong promoter present on the T-DNA inhibited plant tumor formation. This toxic property was tested for application in cell ablation experiments by expressing VirD5 specifically in tapetum cells using a double inducible system consisting of the GAL4/UAS element in combination with the CRE/lox cassette.

Chapter 5 shows that VirD5 also inhibited mammalian cell division. Aurora kinases play essential roles in regulating mitosis and are strongly conserved in different eukaryotes. We found that VirD5 interacted with the three human Aurora kinases and exclusively bound to the catalytic domain of the Ipl1/Aurora kinase. VirD5 displayed a dynamic sublocation in human cells from DNA replication foci in interphase to the centrosomes during the mitotic stage. Ectopic expression of VirD5 caused chromosome mis-segregation and micronuclei formation, hallmarks of tumor cells and in the end triggered cell apoptosis.

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Chapter 2

Chromosome mis-segregation in yeast by *Agrobacterium tumefaciens* virulence protein VirD5

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Abstract

Agrobacterium tumefaciens delivers a segment of transferred DNA (T-DNA) as well as effector proteins through a type IV secretion system into host cells. Here, we report that one of these effector proteins, VirD5, has growth inhibitory effects. Its expression in both *Saccharomyces cerevisiae* and *Arabidopsis thaliana* leads to growth inhibition and cell death. This toxicity is conserved among VirD5 proteins from different *Agrobacterium* strains. Using budding yeast as a model organism, we found that VirD5 is present at the yeast centromeres/kinetochores. Toxicity is relieved by deletion of the Spt4 protein, which is also present at the centromeres. VirD5 can interact with Spt4 and in its absence VirD5 is no longer located at the centromeres/kinetochores. The centromere is a specific chromosomal locus required for the assembly of the kinetochore which mediates the accurate separation of the duplicated sister chromatids over daughter cells during mitosis. The expression of VirD5 generates DNA damage and chromosome mis-segregation. These results highlight a novel role of a bacterial virulence protein to hijack host cells through disturbing the essential mitosis process. This may enhance the tumorigenic potential of the bacterium on its natural hosts, dicotyledonous plants.

Introduction

Agrobacterium tumefaciens, a Gram-negative soil bacterium, is capable of infecting a wide variety of dicotyledonous plants in nature, causing crown gall disease (Stachel and Timmerman, 1987; Cleene and De Ley, 1976). In the process of infection, a single-stranded copy of the transferred DNA (T-DNA) segment from the bacterial tumor-inducing (Ti) plasmid, is transferred and integrated into the host genome (Zambryski, Tempe, and Schell, 1989). Expression of the genes present in the T-DNA in transformed plant cells results in uncontrolled cell division and development of a crown gall tumor (Bochum, 1985).

Besides the T-region the Ti plasmid embraces an area called the Virulence region, which contains a set of genes that are essential for virulence of the bacterium and which mediate the processing of the T-DNA and its delivery into host cells (Hooykaas and Beijersbergen, 1994; Gelvin, 2003). The virulence genes are induced in plant sap by phenolic compounds that are recognized by the chemoreceptor VirA (Turk *et al*., 1991). The VirA protein is a histidine kinase that can phosphorylate the transcriptional activator VirG, which in turn can stimulate transcription of the other *vir* genes (Winans *et al*., 1994; Winans, 1991). The VirD2 protein nicks the Ti plasmid bottom strand at 25bp direct repeats flanking the T-region, and thus releases a single-stranded copy, called the T-strand (Ward and Barnes, 1988; Pansegrau *et al*., 1993). VirD2 remains covalently attached to the 5' end of the T-strand and pilots the T-strand into host cells through a type IV secretion apparatus, which is made up of 11 different VirB proteins and the VirD4 coupling protein (Christie and Vogel, 2000; Dürrenberger *et al*., 1989; Mysore *et al*., 1998). Concurrently with the T-strand, several virulence (Vir) proteins including VirE2, VirE3, VirF and VirD5 are translocated into plant cells via the VirB type IV secretion system of the bacterium (Vergunst *et al*., 2000). The single-stranded DNA binding protein VirE2 is thought to coat and protect the T-strand against nucleases in the host cell cytoplasm (Abu-Arish *et al*., 2004; Christie *et al*., 1988; Citovsky, Wong, and Zambryski, 1989). The Nuclear Localization Signal (NLSs) in VirD2 targets the T-strand to the host cell nucleus (Tzfira and Citovsky, 2000). Besides, the interaction of VirE2 with VIP1, a transcription factor harboring a bZIP motif in *Arabidopsis thaliana* facilitates the transport of VirE2 and the T-complex into the nucleus (Tzfira, Vaidya, and Citovsky, 2001). The transported VirE3 protein is also imported into the host cell nucleus, where it interacts with Brp, a TFIIB like transcription factor and stimulates transcription of host genes including *VBF* (Garcia *et al*, 2006; Niu *et al*., 2015). The VirF protein is a host range factor (Hooykaas *et al*., 1984; Melchers *et al*., 1989) which contains an F-box and thus may be incorporated into an Skp1-Cdc53-F-box (SCF) ubiquitin-ligase (E3) complex in the host cells (Schrammeijer *et al*., 2001). The VirF SCF complex is thought to promote the proteolytic degradation of VirE2 and VIP1 (Tzfira, Vaidya, and Citovsky, 2004). This may lead to decoating of VIP1 and VirE2 from the T-strand and may also dampen the defense response by VIP1 in some plant species. An endogenous F-box protein called VBF in *A. thaliana* may take over from VirF. This explained why the simultaneous deletion of VirF and VirE3 led to much stronger attenuation of virulence than seen in the single mutant (García-Rodríguez, Schrammeijer, and Hooykaas, 2006).

Previous studies in our lab demonstrated that VirD5 is a large virulence protein consisting of 833 amino acids embracing two Nuclear Localization Signals (NLSs), and putative helix-turn-helix and helix-loop-helix domains (Schrammeijer *et al*., 2000). The VirD5 protein can

be transferred independently of the T-strand into the host cell via the type IV secretion system (Vergunst *et al*., 2005). Recently, Magori and Citovsky (2011) suggested that VirD5 stabilizes VirF in host cells via interaction with each other, but Wang *et al* (2014) described VirD5 as a competitor of VBF for binding to VIP1 to stabilize VIP1 and VirE2.

In this report, we used *Saccharomyces cerevisiae* as a model organism to study the function of VirD5. We found that VirD5 binds to the centromeres/kinetochores in the nucleus and interacts with kinetochore-associated protein Spt4, which is also present at the centromeres/kinetochores and plays a role in chromosome segregation (Basrai *et al*., 1996; Crotti and Basrai, 2004). We found that the presence of VirD5 leads to chromosome mis-segregation.

Results

Expression of VirD5 inhibits growth of Arabidopsis thaliana

Previous work in our lab has shown that VirD5 is an effector protein which is translocated into plant cells during infection by *Agrobacterium* (Vergunst *et al*., 2005). In order to obtain more insight into the function of VirD5 we aimed to express the protein in *Arabidopsis thaliana*. To this end, a binary vector containing the *virD5* gene driven by a tamoxifen inducible promoter was transformed into *A. thaliana* via flora dip. Fifteen independent transformed plants were propagated on kanamycin selection medium. In order to test whether VirD5 can influence plant growth and development, T2 seeds of each of these lines were germinated on kanamycin medium to which tamoxifen has been added at 1μM or 10 μM to induce the expression of VirD5. In the presence of tamoxifen seedlings died within 2 weeks, but without tamoxifen the transgenic seedlings showed normal growth (**Figure 1A**). This suggests that VirD5 might target an essential cellular process.

VirD5 inhibits growth of yeast

The yeast *S. cerevisiae* is an excellent model to analyze the function of bacterial effector proteins that in nature exert their function in multicellular eukaryotes. When expressed in yeast a negative effect on the growth of yeast is not uncommon, but this sensitive and measurable phenotype can be exploited in yeast to reveal more about the biological role (Alto *et al*., 2006; Mulla, Zhu, and Li, 2014). To determine if we can take advantage of the yeast system, the *virD5* gene was cloned into a yeast multi-copy plasmid behind the galactose inducible GAL1 promoter and was transformed into strain BY4743. Transformed cells were grown on MY medium containing glucose for 3 days, and thereafter colonies were taken from the plates, suspended and serially diluted and spotted onto an MY plate containing either 2% glucose or 2% galactose which were incubated and grown for additional 3 days. Expression of VirD5 led to growth inhibition also in yeast (**Figure 1B**). This toxic property was highly conserved among VirD5 proteins from different *Agrobacterium* strains (**Figure 1B**). To find out which part of VirD5 is essential for the toxicity, several truncations, but also the full length VirD5 were expressed in yeast strain pJ694A as in frame fusions with the GAL4 binding domain of pAS2.1 vector (CLONTECH), allowing in a subsequent step to search for interaction partners in a yeast 2-hybrid assay. Three days after incubation, presence of the construct embracing full length VirD5 had prevented growth, but presence of neither the N-terminal nor the C-terminal region alone led to a complete inhibition of yeast

growth. However, presence of the N-terminal part of VirD5 led to a delay of growth in contrast to the extreme C-terminal part of VirD5 (**Figure 1C**). These results suggest that both parts together are needed to stop yeast growth. At the same time this allows for their separate use as baits in 2-hybrid screens for interaction partners. In order to find potential interactors, we first used the large N-terminal VirD5 (1-715) or VirD5NT (1-505) fragment fused with GAL4-BD in a yeast two hybrid (Y2H) assay. Unfortunately, both parts of VirD5 showed auto-transcriptional activation activity (data not shown), which was also recently reported by another group (Wang *et al*., 2014) and could therefore not be used in the yeast 2-hybrid screen. Transcriptional activation activity was not completely unexpected as our previous bioinformatics analysis had already shown that VirD5 contains several DNA binding motifs (Schrammeijer *et al*., 2000). Subsequently we used the C-terminus of VirD5 (716-833) lacking the transcriptional activation domain as a bait in a Y2H screen with *A. thaliana* cDNA library and obtained one zinc finger protein (At1g75710) that could bind to the C-terminal part of VirD5. However, binding could not be confirmed in an *in vitro* pull-down assay (data not shown).

Genome-wide deletion library screening

As the toxic effects of VirD5 in its natural host, plants, were recapitulated in yeast (**Figure 1A** and **B**), we used the yeast model organism to dissect the function of VirD5. First of all a genomic deletion library was used in a screening for deletion mutations that suppress the toxic effects of VirD5 as such suppressors may reveal the identity of the target of VirD5. The homozygous diploid deletion collection consists of around 5000 strains, and each strain contains a deletion of a non-essential annotated yeast open reading frame (ORF). A plasmid containing the *virD5* gene under the control of the GAL1 promoter (pMVHis-VirD5) was transformed into all of the deletion strains. After growing on MYglu plates for 3 days, colonies were scratched onto MYgal plates and incubated for an additional 3 days. Most of these transformants cannot survive on MYgal plates due to the lethality of VirD5, but 33 deletion mutants survived. Upon re-analysis of these individual deletion mutants, 11 showed a robust suppression of the toxicity of VirD5 (**Figure 1D** and **E**, **Table 1**). In two of these genes were affected compromising the transcriptional activation of the GAL genes and thus in these expression of VirD5 was prevented, explaining their survival. This also shows the effectiveness of the selection strategy. In the other nine deletion strains that showed a robust suppression of the toxicity of VirD5 different genes were deleted, the products of which may be a potential target of VirD5, may stabilize or enhance the level of VirD5 in the cell, may influence the location of VirD5 in the cell or otherwise may be necessary for the toxicity of VirD5.

To confirm that the deletions in these nine strains were responsible for the suppression of the lethality of VirD5, a complementation assay was performed. The nine wild type genes including their promoter and terminator regions were obtained from the parental strain BY4743 by PCR and cloned into the single-copy yeast vector pRS315. These plasmids were transformed together with pMVHis-VirD5 into the nine strains that were insensitive to VirD5 and then it was tested whether they had become sensitive to VirD5 again. None of the transformants survived on MY plates containing 2% galactose (**Figure 1E**), which demonstrated that deletion of these nine genes is responsible for the suppression of VirD5

toxicity. The products of these genes have been shown to be involved in different complex pathways (**Table 1**).

Subcellular localization of VirD5

In order to gain more insight into the mechanism of the toxicity of VirD5, we studied where VirD5 is localized in *S. cerevisiae*. To answer this question the VirD5 protein was fused N-

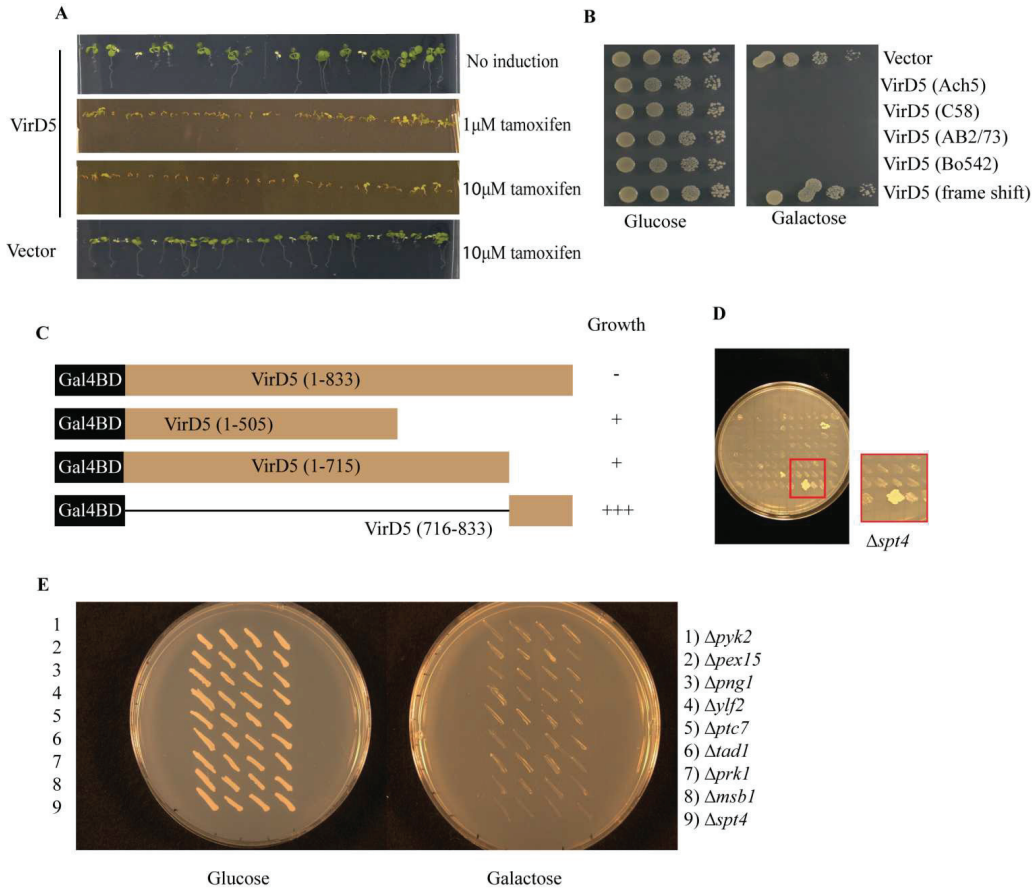

Figure 1. VirD5 inhibits the growth of yeast and plants. (A) Inhibition of growth of transgenic *A. thaliana* expressing VirD5 in the presence of tamoxifen. (B) Yeast cells (BY4743) transformed with plasmid encoding VirD5 from different *Agrobacterium* strains under the control of the GAL1 promoter. Transforment were serially diluted and spotted onto selection medium containing either glucose or galactose. (C) Scheme of the different VirD5 truncations fused in frame with the GAL4 binding domain driven by the constitutive ADH1 promoter that were used to assay for growth inhibition. (D) The whole genome-wide deletion library screening (~5000 strains). All individual deletion mutants transformed with pMVHis-VirD5 were plated on glucose medium first and then spotted onto galactose plates. The Δ*spt4* deletion strongly suppressed the toxicity of VirD5 (enlarged image). (E) Complementation by the wild type genes made the nine mutants shown sensitive again to VirD5.

terminally with the green fluorescent protein (GFP) and expressed under the control of the MET25 promoter in strain BY4743:HTA2-CFP, in which the nucleus was marked by labelling of histone H2A with Cyan fluorescence. The expression of GFP-VirD5 was blocked by the presence of methionine, but one hour after removal of methionine, cells showed green fluorescence under the confocal microscope. While GFP fluorescence was present all over the cell in control cells expressing unfused GFP (**Figure 2A**, a-d), GFP-VirD5 was seen clustered as bright GFP dots in the nucleus (**Figure 2A**, e-h), indicating that VirD5 is localized at specific foci in the nucleus. The genome-wide deletion mutants screening taught that deletion of *SPT4* disrupted the lethal activity of VirD5. Crotti and Basrai (2004) showed that a SPT4-GFP fusion protein is localized to three to seven foci in the yeast nucleus, a pattern resembling that seen with the GFP-VirD5 fusion. Some of the SPT4-GFP foci have been shown to overlap with kinetochore-containing NDC10-HA foci, indicating that a subset of SPT4-GFP foci localize at the kinetochores, where SPT4 contributes to the formation of the centromeric chromatin structure and to chromosome transmission fidelity (Crotti and Basrai, 2004). This suggests that VirD5 might similarly be targeted to centromeres/kinetochores and that its toxicity may be due to impaired chromosome segregation.

In order to find out whether VirD5 like Spt4 may be localized at the kinetochores, the centromere/kinetochore-associated protein Ndc10 and Spt4 were fused with the C-terminus of CFP in a construct driven by the MET25 promoter and subsequently cotransformed with a construct expressing GFP-VirD5 into yeast. Cells were observed under the confocal microscope one hour after the removal of methionine. GFP-VirD5 foci overlapped fully with both CFP-Spt4 foci (**Figure 2B**, a-d) and CFP-Ndc10 foci (**Figure 2B**, e-h), suggesting that VirD5 like Spt4 is present at the centromeres/kinetochores that are marked by Ndc10.

The N-terminal part of VirD5 is targeted to the kinetochores/centromeres

It was shown by previous bioinformatics prediction that VirD5 is made up of 833 amino acids and contains several functional motifs (Schrammeijer *et al*., 2000). In order to find out which part of VirD5 mediates targeting to the centromeres/kinetochores in yeast cells, the N-terminal 505 amino acids of VirD5 (VirD5NT) and the C-terminal 313 amino acids of VirD5 (VirD5CT) fused in frame with the C-terminus of GFP were expressed under the control of the MET25 promoter in wild type BY4743 cells. After shifting to methionine free medium for 1 hour, a GFP dot was only seen in the nuclei of cells expressing GFP-VirD5NT, but not in those expressing GFP-VirD5CT, where the GFP signal was distributed all over the cell (**Figure 3A**). This indicates that the N-terminus of VirD5 mediates the accumulation at the centromeres/kinetochores. We also made a construct embracing a smaller N-terminal part, VirD5 (1-202). In contrast to VirD5 (1-505) this construct did not accumulate at the centromeres/kinetochores. Subsequently, we verified the growth-inhibitory properties of these constructs. To test this, constructs encoding VirD5 (1-202) and VirD5 (1-505) driven by the GAL1 promoter were introduced into BY4743 yeast cells. As can be seen in **Figure 3B**, the expression of VirD5 (1-505) led to growth inhibition, but the expression of VirD5 (1-202) did not interfere with growth. These results are in line with the previous findings indicating that targeting to the centromeres/kinetochores is necessary to effect growth inhibition.

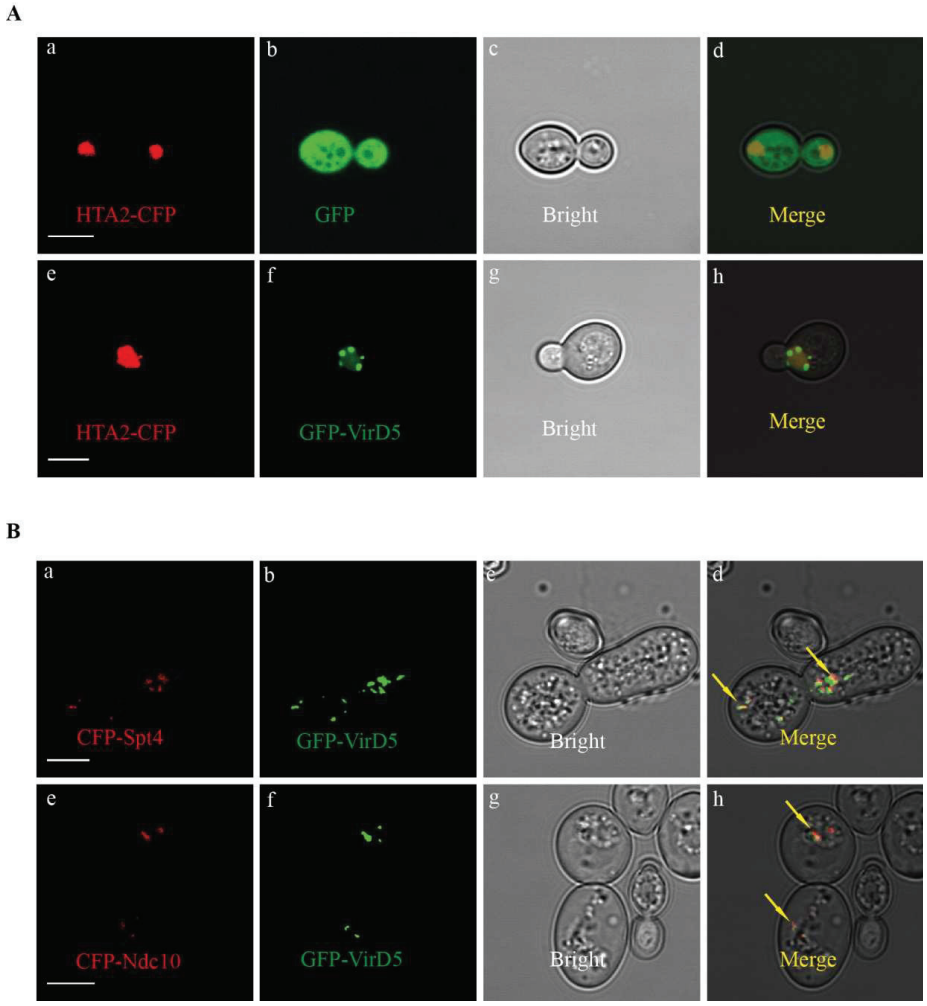

Figure 2. Localization of VirD5 in foci in the nucleus and co-localization with the kinetochores. (A) Yeast cells (BY4743-HTA2-CFP) transformed with plasmids encoding empty GFP (a-d) and GFP-VirD5 (e-h). HTA2-CFP represents the histone HTA2 fused with CFP and marks the nucleus. (B) Yeast cells transformed with plasmid encoding GFP-VirD5 together with plasmid encoding either CFP-Spt4 (a-d) or CFP-Ndc10 (e-h). Yellow arrows indicate the overlaps. Scale bar, 5 μm.

VirD5 physically interacts with Spt4

Spt4 is a functional and structural component of the centromeric loci, and is required for the integrity of centromeric chromatin (Crotti and Basrai, 2004) and deletion of *SPT4* suppressed the lethality of VirD5 (**Figure 1D** and **E**). We thus wondered whether VirD5 could physically bind to Spt4 in yeast cells. To test this, we performed Bimolecular Fluorescent Complementation (BIFC) experiments (Kerppola, 2008). VirD5 was fused with the C-

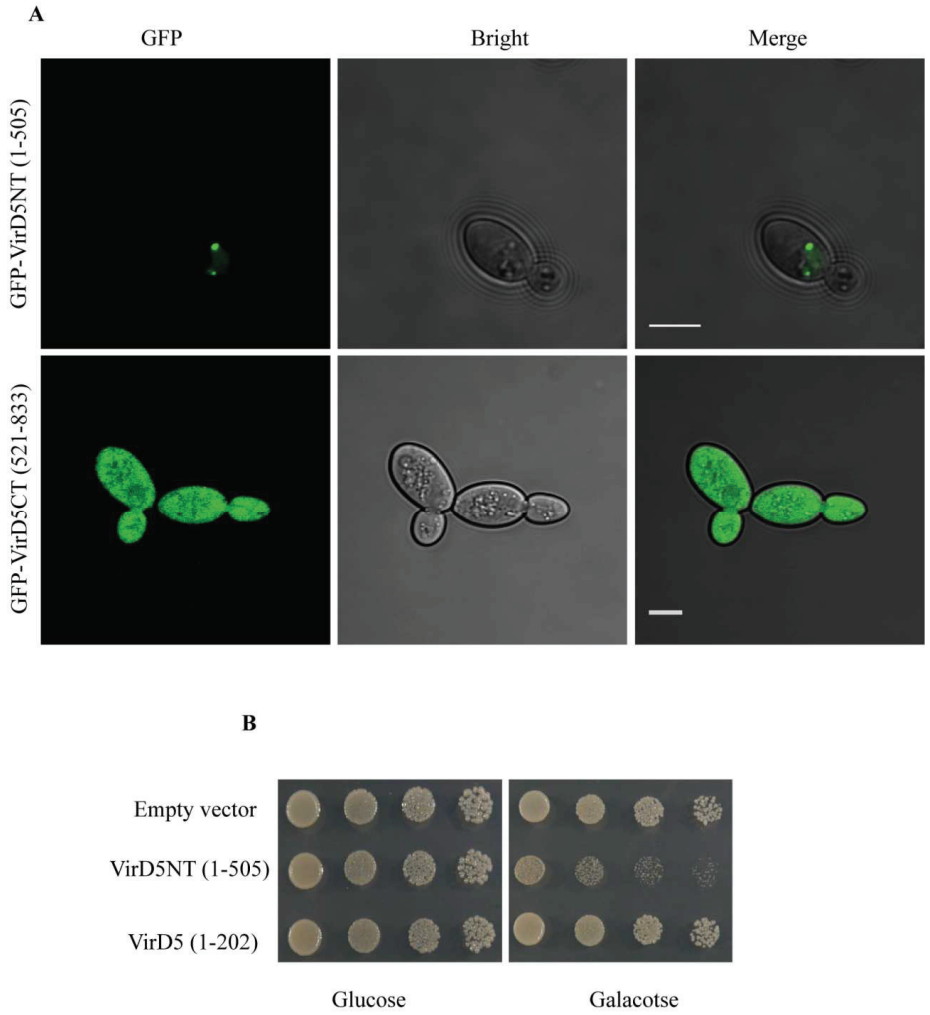

Figure 3. The N-terminus of VirD5 is targeted to centromeres/kinetochores in the nucleus. (A) Yeast cells (BY4743) transformed with plasmid encoding either GFP-VirD5NT (1-505) or GFP-VirD5CT (521-833). Scale bar, 5 μm. (B) Yeast cells (BY4743) transformed with either empty high copy plasmid (pRS425) or plasmid encoding VirD5NT (1-505) or VirD5 (1-202) under the control of the GAL1 promoter. Transformants were serially diluted and spotted onto selection medium containing either glucose or galactose. VirD5NT (1-505), the N-terminal 505 amino acids of the VirD5 protein. VirD5CT (521-833), the C-terminal 313 amino acids of the VirD5 protein. VirD5 (1-202), the N-terminal 202 amino acids of the VirD5 protein.

terminal part of YFP (VC173) and transformed into BY4743 cells together with Spt4 fused with the N-terminal part of YFP (VN173). As can be seen in **Figure 4A** (upper panel), VirD5 displayed a very strong BIFC signal with Spt4 in the nucleus, whereas the fusions of VirD5 or Spt4 introduced together with unfused complementary part did not give a YFP signal (**Figure 4A**, middle and lower panel). To confirm this interaction, an *in vitro* pull-down assay

was performed as follows: GST or GST-Spt4 was expressed in *E.coli* and bound to the Glutathione HiCap Matrix as the bait. The beads were incubated separately with His-tagged VirD5 purified from *E.coli* for 2 hours at room temperature in binding buffer containing 0.1%

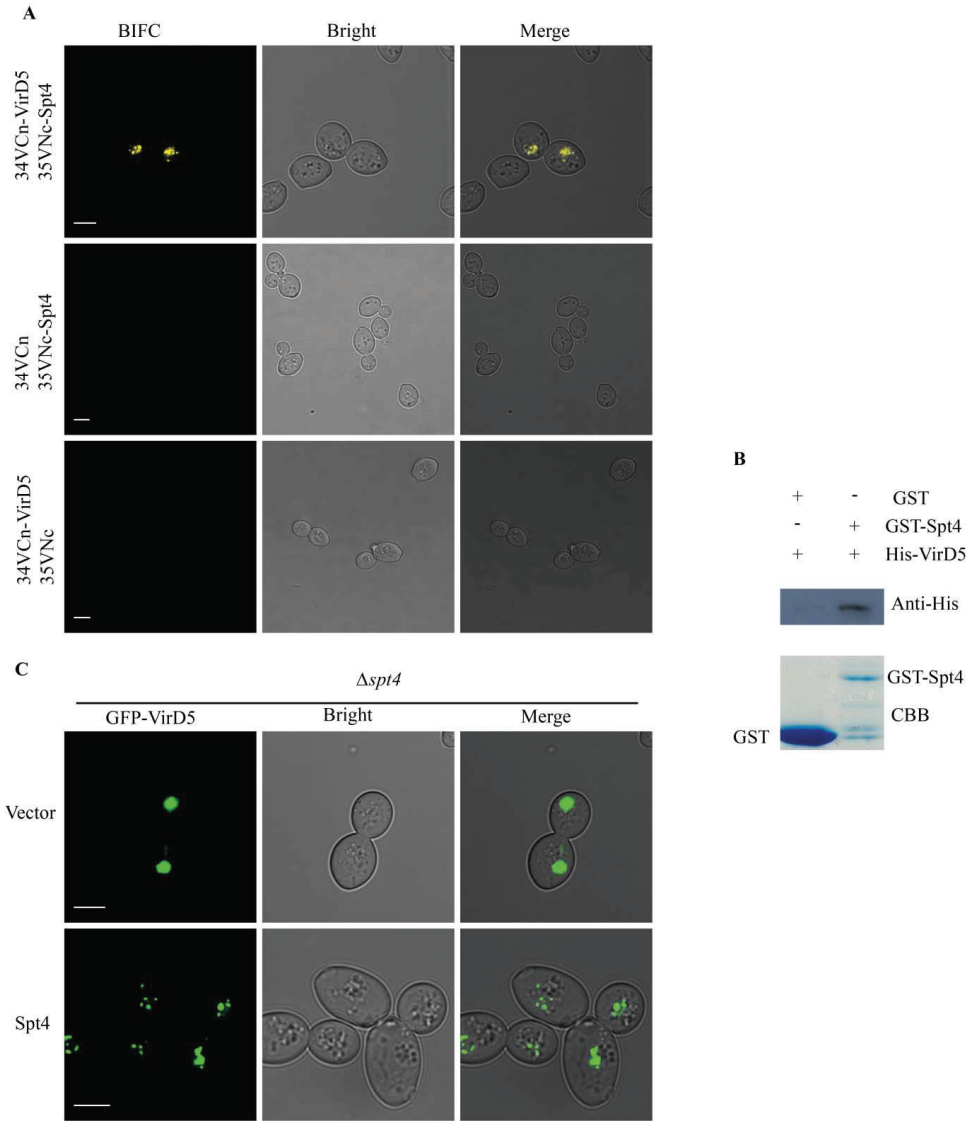

Figure 4. VirD5 physically interacts with Spt4. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP (VC173) fused with the N-terminus of testing proteins. 35VNc, the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. Scale bar, 5 μm. (B) His-tagged VirD5 purified from *E.coli* was incubated with either empty GST or GST-Spt4; after washing steps, the presence of His-VirD5 was detected by anti-His antibody. Lower panel, CBB staining of GST and GST-Spt4. (C) Plasmid encoding GFP-VirD5 alone or with plasmid encoding wild type Spt4 was transformed into Δ*spt4* cells. Scale bar, 5 μm.

Triton-100. After 3 times washing, the protein mixtures were separated on a 10% SDS-PAGE gel. As shown in **Figure 4B**, VirD5 physically interacted with GST-tagged Spt4, but not with empty GST, suggesting that Spt4 might be a direct target of VirD5.

Above we identified nine deletion mutations that completely suppressed the lethality of VirD5. We were therefore interested to find out whether any of these mutations led to an altered localization of VirD5 in the cell. To this end GFP-VirD5 was transformed into these nine deletion mutants, and transformants were observed under the confocal microscope. In eight of the mutants the nuclear GFP foci could still be observed, but in the *spt4* deletion strain no foci were present in over 90% of transformed cells, but GFP was present all over the nucleus (**Figure 4C**, upper panel). Only a few foci were present in the remaining 10% of transformed cells. When the wild type *SPT4* gene was cotransformed with GFP-VirD5 into the *spt4* deletion strain the punctate foci of VirD5 were again observed (**Figure 4C**, lower panel). These data indicate that Spt4 binds to VirD5 and thus localizes it at the centromeres/kinetochores, allowing to exert its toxic effect at the centromeres/kinetochores. Alternatively, Spt4 might function as a molecular chaperon that facilitates VirD5 to fold into a correct conformation. Finally, Spt4 might help create a local chromatin that allows VirD5 to bind to the centromeres/kinetochores to exert its toxic effect.

VirD5NT causes sensitivity to benomyl

Kinetochores are large protein complexes that assemble exclusively on the centromeric regions of the chromosomes and interact with spindle microtubules to mediate the separation of the paired sister chromatids over daughter cells during mitosis. Both the full length VirD5 and VirD5NT were localized at the centromeres/kinetochores (**Figure 2** and **3A**) and inhibited yeast growth (**Figure 1B**, **C** and **3B**). We wondered whether targeting the centromeres/kinetochores by VirD5 may affect mitosis in yeast cells. To this end, we examined the sensitivity of cells expressing VirD5NT to benomyl, a microtubule-depolymerizing drug for which kinetochore mutants are hypersensitive. As can be seen in **Figure 5A**, yeast BY4743 cells transformed with either empty single-copy (pRS315) or high-copy (pRS425) vector showed a mild sensitivity to benomyl. However, yeast cells expressing both high and low levels of VirD5NT were heavily compromised in growth in the presence of benomyl, indicative of benomyl hypersensitivity like kinetochore mutants.

The yeast Spt4 protein is not only abundant at the kinetochores, but also associated with HMRa and telomeres (TEL) loci and plays a role in gene silencing at these heterochromatic loci (Crotti and Basrai, 2004). In view of the direct binding of VirD5 to Spt4 it is thus possible that VirD5 is also present at these loci. Since VirD5NT (1-505) has putative DNA binding domains and confers transcriptional activation (Wang *et al*., 2014; data not shown), its presence at these heterochromatic loci might affect heterochromatic gene silencing. In order to test this two yeast strains containing a silent *URA3* reporter gene inserted adjacent to either HMRa (BUY545) or telomeres (TEL) (BUY668) were transformed with either an empty high-copy plasmid or the same plasmid encoding VirD5NT (1-505) controlled by the GAL1 promoter. The expression of *URA3* can be estimated by growth on medium with 5-Fluoroorotic acid (5-FOA), which is converted into the toxic 5-Fluoro-uracil, when *URA3* is expressed and thus inhibits yeast cell growth (Boeke, Lacroute, and Fink, 1984). Yeast cells containing the empty vector showed only a slight growth inhibition in the presence of 5-FOA,

indicative of the repression of *URA3*, whereas cells expressing VirD5NT (1-505) displayed a strong growth inhibition in the presence of the drug (**Figure 5B**). This indicates that by a direct interaction with Spt4, VirD5 interfered with heterochromatinization, possibly by its N-terminal transcriptional activation functions.

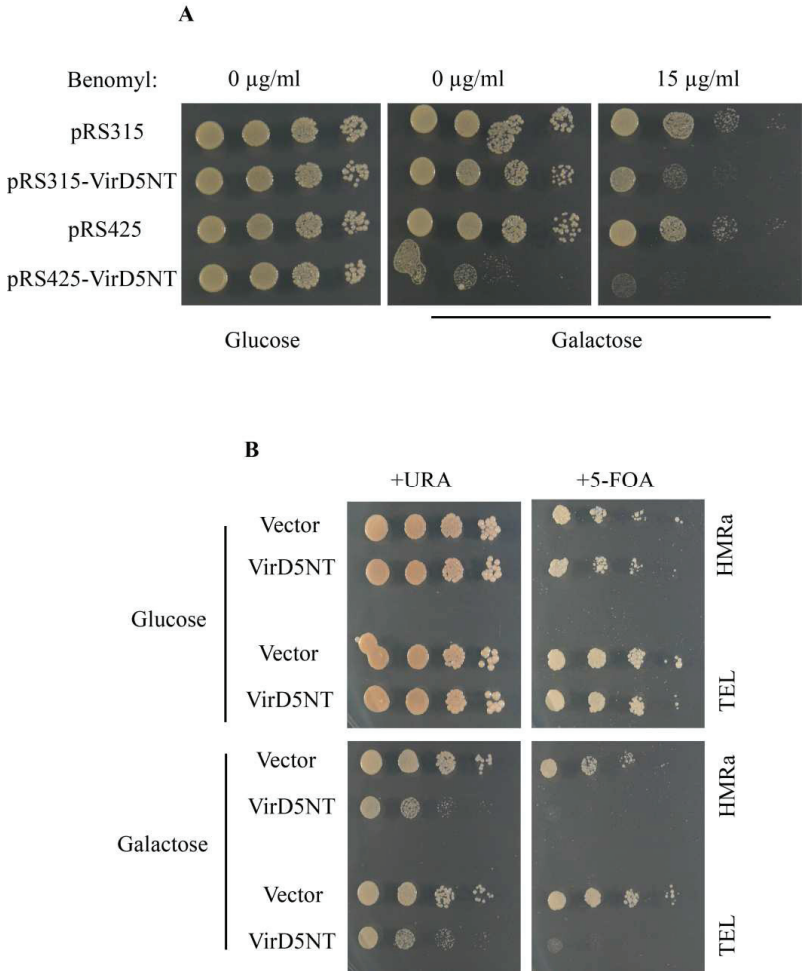

Figure 5. Presence of VirD5NT leads to benomyl hypersensitivity and defective gene silencing at HMRa and TEL loci. (A) Yeast (BY4743) cells were transformed with either empty single-copy plasmid (pRS315) or high-copy plasmid (pRS425) or plasmid encoding VirD5NT driven by the GAL1 promoter. Transformants were serially diluted and spotted onto minimal media containing either glucose or galactose with or without benomyl. (B) The BUY545 strain contains a *URA3* reporter gene adjacent to HMRa and BUY668 contains a *URA3* reporter gene integrated adjacent to one of the telomeres (TEL). Cells were transformed with either empty high-copy plasmid or the same plasmid expressing VirD5NT from the GAL1 promoter. Transformants were serially diluted and spotted onto minimal media containing glucose or galactose with or without 5-FOA and incubated for 3 days. VirD5NT, the N-terminal 505 amino acids of VirD5.

Chromosome mis-segregation in the presence of VirD5

During mitosis, the replicated chromosomes are distributed with high fidelity over the daughter cells by the spindle. For this to occur accurately the kinetochores of each pair of chromatids need to be linked to a microtubule that is linked to a different spindle pole. Improper binding (for instance both kinetochores of a pair to microtubules linked to the same spindle pole) can result in chromosome mis-segregation and aneuploidy (Cimini, 2008; Grancell and Sorger, 1998; Thompson and Compton, 2011). In view of its localization at centromeres/kinetochores and its binding to the kinetochore protein Spt4 we wondered whether VirD5 induces chromosome mis-segregation in yeast cells. In order to test this, we expressed VirD5 driven by the GAL1 promoter in HTA2-CFP marked yeast cells (BY4743:HTA2-CFP), and found that most cells displayed a large elongated bud and failed to segregate their chromosomes equally to daughter cells at anaphase in the presence of galactose (**Figure 6A**, lower panel), while wild type HTA2-CFP marked cells showed a normal chromosome distribution (**Figure 6A**, upper panel).

In order to examine whether the presence of VirD5 would lead to the formation of aneuploid cells, we measured the DNA content of cells expressing VirD5 using flow cytometry. Cells growing in medium containing glucose or galactose, respectively, were harvested and treated for measurement. As shown in **Figure 6B**, the peaks representing the DNA content were shifted to a lower DNA content in the cells that had been growing in medium containing galactose, but the ratio between n-like and 2n-like content had shifted to 2n. All this suggested that many cells had become diploid and most of the cells had become aneuploid.

To gain a further confirmation that VirD5 causes chromosome mis-segregation, we carried out the following chromosome loss assay. In this experiment, we used yeast strain RLY4029 (Chen *et al*., 2012), which contains a chromosome fragment (CF) harboring the *URA3* gene and the *SUP11* gene suppressing red pigment accumulation as a consequence of the chromosomal ade2-101 mutation. Cells carrying CF produce white colonies, whereas cells lacking CF form red colonies. RLY4029 cells with and without a construct encoding VirD5 under the control of GAL1 promoter inserted at the *LEU2* locus were grown in minimal medium containing glucose but lacking uracil first, followed by a shift to rich medium (with uracil) containing 2% raffinose and 2% galactose for 24 hours. The induced cells were serially diluted and plated on rich media containing glucose. As seen in **Figure 7A**, a more than 10-fold higher rate of mini-chromosome loss was observed in VirD5 expressing cells compared with that in control cells. These data strongly indicate that VirD5 causes chromosome instability.

VirD5 triggers DNA damage

Chromosome mis-segregation, chromosome instability and aneuploidy are commonly observed in human cancer cells and frequently lead to DNA damage (Janssen *et al*., 2011). We therefore sought to find out whether chromosome mis-segregation induced by VirD5 may also be accompanied by DNA damage. To test this, we carried out a Clamped Homogeneous Electrical Field (CHEF) electrophoresis assay. Intact chromosomes from wild type and cells with a chromosomally integrated construct encoding VirD5 driven by the GAL1 promoter were isolated and separated in a CHEF gel. As can be seen in **Figure 7B**, a massive DNA

smear was observed in VirD5 expressing cells, but not in wild type cells, illustrating that VirD5 causes chromosomal fragmentation.

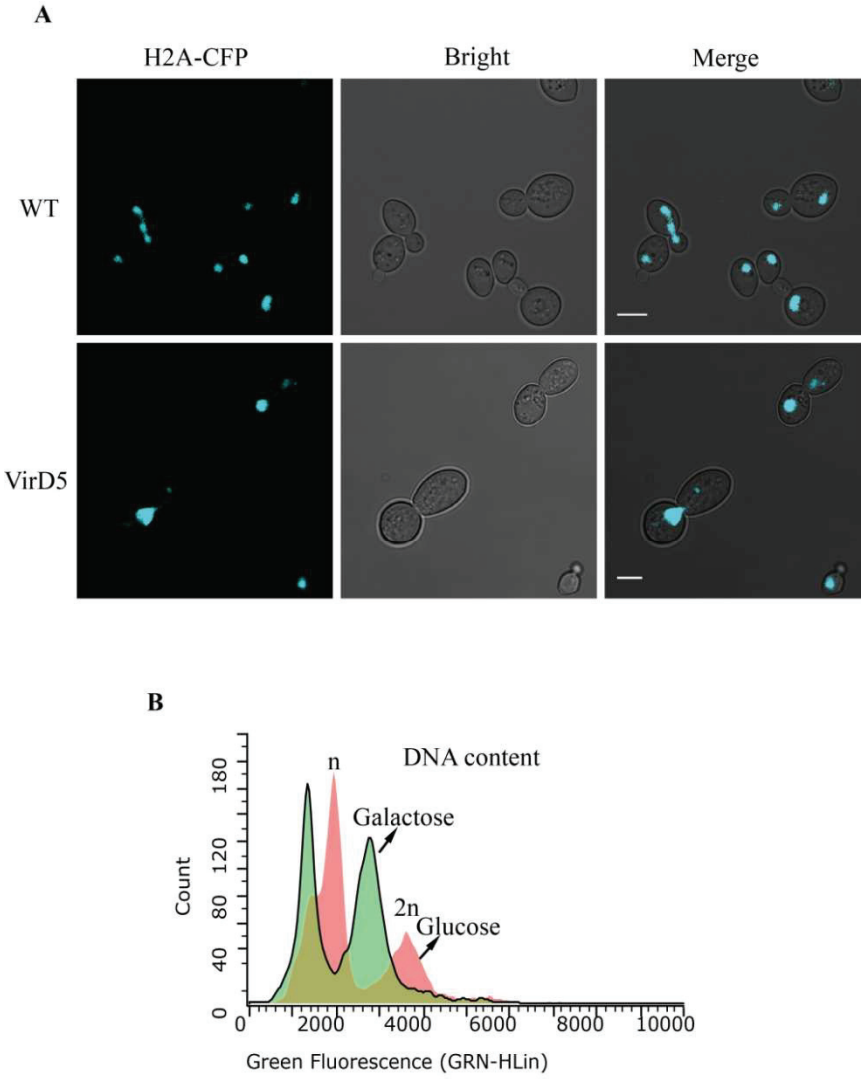

Figure 6. VirD5 disturbs chromosome segregation. (A) The HTA2-CFP (Histone 2A) marked strain (BY4743:HTA2-CFP) with or without integration of *virD5* driven by the GAL1 promoter. After switching to galactose containing medium, a CFP signal was detected with the confocal microscope. Scale bar, 5 μm. (B) Yeast cells (BY4743:pGAL1-VirD5) with a chromosomally integrated construct encoding VirD5 driven by the GAL1 promoter were cultured in rich media containing either glucose (red) or galactose (green), followed by fixation and flow cytometer measurement.

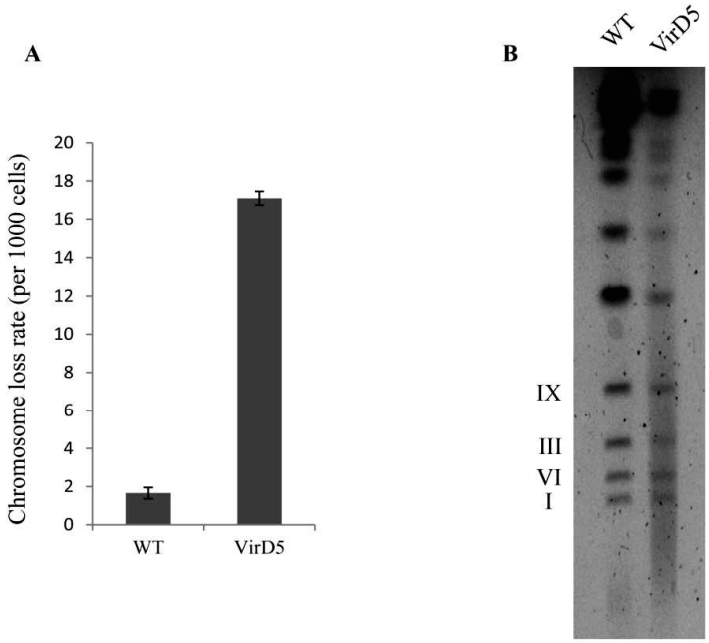

Figure 7. VirD5 causes massive DNA damage. (A) The yeast strain RLY4029 contains an artificial minichromosome harboring a gene (*SUP11*) suppressing red pigment accumulation. The chromosome loss rate was inferred from the frequency of red colonies. Error bars represent the mean ± SD from three independent experiments. (B) Chromosomes from yeast strain BY4743 and its derivatives harboring pGAL1-VirD5 were separated in a CHEF gel. All experiments were repeated at least three times.

Discussion

A. tumefaciens delivers T-DNA and several different virulence proteins into host cells during infection, amongst which the VirD5 protein (Vergunst *et al*., 2005). While the precise function of this protein is still elusive up to date, two groups have described VirD5 either as a competitor of VBF for binding to VIP1 thus stabilizing VIP1 and VirE2 or reversely as stabilizing VirF thus promoting degradation of VIP1 and VirE2 in host cells (Magori and Citovsky, 2011; Wang *et al*., 2014). To gain further insights into the functions of this protein, we have used budding yeast as a model organism. Budding yeast has been exploited before as an excellent system to study the function of bacterial effector proteins. Alto and colleagues (Alto *et al*., 2006) have used yeast to demonstrate that effectors IpgB1 and IpgB2 from *Shigella* subvert host cells via mimicking Rho family small G proteins. Kramer and coworkers (Kramer *et al*., 2007) have screened a haploid yeast deletion strain collection to identify the function of the *Shigella* effector OspF as an inhibitor of MAPK signaling. Although unicellular yeast is not a natural host of *Agrobacterium*, previous studies in our lab (Bundock *et al*., 1995) have shown that *Agrobacterium tumefaciens* also can transfer T-DNA and effectors into *Saccharomyces cerevisiae*.

Our results showed that VirD5 inhibited the growth of both plant and yeast cells (**Figure 1A** and **B**), and this inhibitory activity was highly conserved among VirD5 proteins from different *Agrobacterium* strains. These observations suggested that VirD5 might target a conserved essential process in both yeast and plant. Thus, yeast seemed a suitable organism to determine the potential roles of VirD5. Our first genome-wide deletion library screening demonstrated that thirty three deletion mutants suppressed the lethality of VirD5. However, only nine of these showed a robust growth in the presence of VirD5 (**Table 1**), but functions of these nine deleted genes did not immediately hint at the role played by VirD5. Interestingly, GFP-VirD5 expression in yeast cells displayed specific punctate foci mostly in the nuclear membrane (**Figure 2A**, e-h). A similar pattern was seen previously in cells expressing a SPT4-GFP fusion protein (Crotti and Basrai, 2004). As deletion of SPT4 suppressed the lethal activity of VirD5, this motivated our focus on the protein Spt4, a transcription elongation factor, which forms a heterodimeric complex with Spt5 to regulate mRNA transcription via direct interaction with RNA polymerase II. A gene for this highly conserved protein is present in the human genome, as well as that of yeast, plants and other eukaryotes (Dürr *et al*., 2014; Hartzog *et al*., 1996; Wada *et al*., 1998). The Spt4 protein also plays a role in chromosome segregation and is a functional and structural component of centromeric heterochromatin (Basrai *et al*., 1996; Crotti and Basrai, 2004). We found that the deletion of *spt4* in yeast suppressed the lethality of VirD5 (**Figure 1D** and **E**), and further data demonstrated that VirD5 colocalized and physically interacted with Spt4 (**Figure 4A** and **B**). The localization of VirD5 in the cell was altered in the *spt4* mutant: the protein no longer was present at the centromeres/kinetochores in this mutant background (**Figure 4C**), and as a consequence VirD5 was no longer lethal. Presence of VirD5 at the centromeres/kinetochores may be because of a direct interaction between VirD5 and SPT4 or because of the role of SPT4 in the stimulation of transcription at the centromere, bringing about structural changes enabling VirD5 to localize here.

The centromere is a specialized nucleosome that mediates chromosome attachment via the kinetochore to the spindle microtubule. In budding yeast, transcription at the centromere induced by the transcription factor Cbf1, an inner kinetochore protein that binds directly to the centromeric DNA facilitates the centromere function (Ohkuni and Kitagawa, 2011). However, strong transcription over the centromere in budding yeast by locating an artificial strong promoter (GAL1) adjacent to the centromere inactivated its function, thereby inducing chromosome mis-segregation and aneuploidy (Hill and Bloom, 1987). As the VirD5 protein is not only present at centromeres/kinetochores by interaction with Spt4 (**Figure 2** and **4C**), but also has transcriptional activation activity (Wang *et al*., 2014; data not shown), it is possible that the toxic effects of VirD5 are due to erroneous transcription at the centromeres.

We found that the presence of VirD5 leads to chromosome mis-segregation and aneuploidy and massive DNA damage (**Figure 6** and **7**). *A. tumefaciens* causes crown gall tumor formation on plants by inserting an oncogenic DNA segment in the plant chromosomes. Although absence of the *virD5* gene does not have a strong impact on tumor initiation, we suspect that VirD5 may still contribute to tumor initiation and development in two ways. First of all, DNA breaks may form entry sites for T-DNA integration. Secondly, the generation of aneuploidy cells and cells with chromosome mutations may create the cell

variability that allows evolution of fast growing tumor cells. Such alterations in chromosome content have been correlated with tumor formation in humans.

Materials and Methods

Plant material

Binary vector pGPINTAMVirD5 containing *virD5* under the control of a tamoxifen inducible promoter was transferred into *A. tumefaciens* strain AGL1 via triparental mating (Ditta *et al.*, 1980). *A. thaliana* ecotype Columbia-0 (Col-0) was used for floral dip. A few weeks after dipping, mature seeds were harvested and sowed on MS medium containing 50 mg/L kanamycin. Kanamycin resistant T1 transgenic seedlings were checked for the insert by PCR and transferred to soil. T2 seeds from 15 independent T1 transgenic plants were germinated on MS media containing kanamycin and either DMSO or different concentration of tamoxifen to induce the expression of VirD5.

Yeast deletion library screening

The complete collection (~5000 mutants) of homozygous diploid deletion strains of BY4743 was purchased from Euroscarf. Cells were taken from original 96-wells plates and cultured in new 96-wells plates containing 200 μl YPD with G418 (150 μg/ml) at 30 ^{o}C with continuous shaking at 700 rpm for 48 hours. Then 20 μl samples from these cultures were transferred into new 96-wells plates containing 180 μl YPD with G418 (150 μg/ml) and grown for 4 hours at 30 ^{o}C with continuous shaking at 700 rpm. After that cells were harvested by centrifugation at 4000 rpm for 3 minutes and suspended in 100 μl 100 mM LiAc, followed by additional centrifugation at 4000 rpm. The supernatants were discarded and pellets from each well were resuspended in 200 μl premixed solution (240 μl 50% 3350PEG, 36 μl 1 M LiAc, 25 μL ssDNA and 100 ng pMVHis-VirD5 plasmid), followed by incubation at 30 ^{o}C for 30 minutes and subsequent heat shock at 42 ^{o}C for 20 minutes. Cells were centrifuged again at 4000 rpm for 3 minutes and resuspended in 40 μl water. Finally 20 μl resuspended cells were spotted onto MY plates containing 2% glucose, histidine and leucine. After 3 days, colonies from selective glucose plates were picked and scratched onto MY plates containing 2% galactose, histidine and leucine and grown for 3 days.

Subcellular localization of VirD5

Plasmids pUG34GFP and pUG34GFP-VirD5 were transformed into yeast BY4743 cells. Transformants were grown at 30 ^{o}C on solid MY media containing methionine to suppress the expression of VirD5. Three days after transformation, colonies were transferred to MY liquid medium containing methionine. Overnight cultures were diluted and grown at 30 ^{o}C in fresh MY liquid medium lacking methionine to induce the expression of VirD5 for 1 hour. Cells were collected by centrifugation for the observation of the GFP signal (excitation, 488 nm; emission, 520 nm) using a 63xoil objective on the Zeiss Imager confocal microscope. Images were processed with ImageJ (ImageJ National Institutes of Health). Plasmids and yeast strains used in this study are listed in **Table 3** and **4**, respectively.

BIFC assay

The pUG34VCn-VirD5 plasmid and empty vector pUG34VCn were transformed either with pUG35VNc-Spt4 or pUG35VNc into yeast cells. Transformants were grown at 30 ^{o}C on solid MY medium containing methionine to inhibit the expression of VirD5. After 3 days, colonies were transferred to MY liquid medium containing methionine. Overnight cultures washed twice with sterilized water were transferred into new flasks containing MY medium lacking methionine to induce the expression of VirD5. After induction for 1 hour, cells were harvested for BIFC signal visualization using a 63xoil objective on the Zeiss Imager confocal microscope. Images were processed with ImageJ (ImageJ National Institutes of Health).

Pull-down assay

GST and GST-Spt4 were expressed in *E.coli* strain Rosette2PLySs. Equal amounts of the GST-tagged proteins were immobilized on Glutathione HiCap Matrix (Qiagen, 30900) for 2 hours at room temperature, followed by a 3 times washing step with washing buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA). The beads were incubated with purified His-tagged VirD5 protein in binding buffer (50 mM NaH_2PO4, pH 7.2, 150 mM NaCl, 0.1% triton X-100) for 2 hours at room temperature. After 3 times washing with buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40), samples were mixed with 20 µL 4x sample buffer and boiled for 10 minutes, followed by centrifugation for 2 minutes at 2000 rpm. Supernatants were loaded to a 10% SDS-PAGE gel for electrophoresis. The presence of the His-tagged VirD5 protein was detected with Anti-His HRP antibodies (Santa Cruz Biotechnology, sc-8036 HRP) by Western Blot analysis.

DNA content measurement

BY4743 yeast cells with the *virD5* gene driven by the GAL1 promoter integrated at the chromosomal *LEU2* locus were grown overnight in rich media containing glucose. Cells were diluted to an OD_{620} of 0.1 and recultured in rich medium containing either glucose or galactose for 6 hours. One ml cells were harvested and fixed overnight with 3.5 ml 100% ethanol at 4 ^{o}C. Samples were washed twice with water and once with sodium citrate buffer. Then 0.5 ml 2 mg/ml RNase (Sigma) solution was added to the cell suspensions. After incubation for 2 hours at 50 ^{o}C, 20 µL 20 mg/ml Proteinase K solution (Qiagen) was added to digest the protein thoroughly at 50 ^{o}C. 1 hour later, samples were mixed with 50 µL SYBR Green I (Sigma) and stored in the refrigerator overnight. The mixture was sonicated and analyzed by a guava easyCyte™ Flow Cytometer (Merck Millipore).

Complementation assay

The *PYK2, PEX15, PNG1, YLF2, PTC7, TAD1, PRK1, MSB1 and SPT4* genes including their promoters and terminators were amplified from yeast strain BY4743. Primers used are listed in **Table 2**. PCR products were purified and digested overnight with NotI and XmaI for the subsequent insertion into NotI/XmaI digested single-copy plasmid pRS315. Positive plasmids were verified by Sanger sequencing. Plasmids containing above genes were transformed into the respective deletion strains together with the high copy plasmid pMVHis containing *virD5* under the control of the GAL1 promoter according to the lithium acetate method. After 3

days on MY glucose selection medium, colonies were restreaked on minimal selection medium containing glucose or galactose and incubated for additional 3 days at 30 ^{o}C.

Chromosome loss assay

Strain RLY4029 (a kind gift from Dr Rong Li) contains a fragment of yeast Chr III, with the *SUP11* and *URA3* marker genes (Chen *et al*., 2012). The genetic background of this haploid strain carries an *ade2-101* mutation and therefore forms red colonies. The red pigment accumulation can be suppressed by the expression of Sup11 present in the minichromosome, resulting in white colonies. The frequency of loss of this minichromosome can therefore be calculated by counting the numbers of red colonies among the total numbers of colonies. *VirD5* under the control of the GAL1 promoter was integrated at the chromosomal *LEU2* locus of strain RLY4029, generating strain RLY4029:*p*GAL1-VirD5 that can grow on MYglu without leucine and uracil. Parental and VirD5 containing yeast cells were cultured overnight in MYglu selection media lacking uracil at 30 °C. Cells were diluted and recultured in MYglu liquid media without uracil for additional 6 hours. After that, cells were diluted 50 folds and switched to rich media containing 2% galactose for 24 hours at 30 °C. Overnight cultured cells were diluted to an appropriate density and plated onto rich media containing 2% glucose for 3 days at 30 °C. Plates were kept at 4 °C for accumulation of red pigment. Total white and red colony numbers were counted.

Separation of chromosomes on CHEF gels

Intact yeast chromosomes were isolated in agarose plugs as described in the CHEF kit (Bio-Rad, 170-3591). A number of $6x10^{8}$ overnight cultured yeast cells were washed twice with 0.1M EDTA (pH 7.5) and resuspended in 630 µL suspension buffer. The suspension mixed with 370 µL 2% low-melt agarose was used to make plugs for CHEF. Plugs were placed in a 1% agarose gel and sealed with liquid agarose. Electrophoresis was carried out in 0.5xTBE at 14 ^{o}C for 24 hours with an initial switch time of 60 s and a final time of 90 s at 200 V. The separated chromosomes were stained with ethidium bromide.

Acknowledgements

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Table 1. Yeast deletion mutations which suppressed the lethality of VirD5.

Yeast mutant	Gene function
apm1	Clathrin-associated protein complex
dbp1	ATP-dependent RNA helicase of the DEAD-box protein
dia3	Hypothetical protein
ent4	Clathrin-mediated endocytosis
gal3	Transcriptional regulator GAL3
gal4	DNA-binding transcription factor required for the activation of the GAL genes in response to galactose
inp1	Peripheral membrane protein of peroxisomes
mdh3	Peroxisomal malate dehydrogenase
mgt1	DNA repair methyltransferase (6-O-methylguanine-DNA methylase)
mlh1	Mismatch repair
mlp1	Myosin-like protein
msb1	Bud development
msh2	Mismatch repair
nup53	Subunit of the nuclear pore complex
pau11	Member of the seripauperin multigene family
pex15	Peroxisomal membrane protein
pex18	Peroxin
png1	Peptide N-glycanase
prk1	Protein serine/threonine kinase
pso2	Nuclease for a post-incision step in the repair of DNA single and double-strand breaks
ptc7	Type 2C protein phosphatase (PP2C)
pyk2	Pyruvate kinase
rps22a	Component of the small (40S) ribosomal subunit
spt4	Transcription elongation, kinetochore and gene silence regulation
tad1	tRNA-specific adenosine deaminase
thp2	Subunit of the THO complex
tif4631	Translation initiation factor eIF4G
tos2	Membrane anchor protein
tpc1	Mitochondrial membrane transporter
vac14	Protein trafficking
vps72	Component of the SWR1 complex
YDR015C	Unknown function
ylf2	GTPase

Table 2. Primers used in this study.

Name	Sequences (5'-3')
Spt4CFPF	CGGGATCCATGTCTAGTGAAAGAGC
Spt4CFPR	CGCGTCGACTTACTCAACTTGACTGC
Ndc10CFPF	CGGGATCCATGAGATCATCGATTTTGTTTC
Ndc10CFPR	CGCGTCGACTCAGTTAGATAGATATACTAACAGACC
Spt4BFW	GGACTAGTCATGTCTAGTGAAAGAGCCTGT
Spt4BREV	ACGCGTCGACGCTCAACTTGACTGCCATCCC
pMVHisVirD5FW	GGACTAGTTCACGCTGGGCGTAACCACCA
pMVHisVirD5REV	ACGCGTCGACATTAAAGCCTTCGAGCGTCCC
MSB1FW	AAAGCGGCCGCACTTATTGATGCAACTGGAGT
MSB1REV	CCCCCCGGGGAATATGGAAAATAAAATGTTA
PNG1FW	AAAGCGGCCGCGCGCTTATAAATTCTCAATC
PNG1REV	CCCCCCGGGGTACAAACAAGCTAGAGAAAATC
TAD1FW	AAAGCGGCCGCTAGGCAGGACAATTTCAGTG
TAD1REV	CCCCCCGGGTGGGGAAATGATAGATGATGG
SPT4FW	AAAGCGGCCGCTCCAATTTACGTGAAGTAGAT
SPT4REV	CCCCCCGGGACCTTTTTTTTCTAATGAAAGTC
PYK2FW	AAAGCGGCCGCCGCTTTTATGAACATATTCCGA
PYK2REV	CCCCCCGGGCTTACCAGACTGTGCGTAAACT
PTC7FW	AAAGCGGCCGCTGAAAATTTGAAAATGTCCTAC
PTC7REV	CCCCCCGGGAACCGAGCGAAACAAGATTA
Pex15FW	AAAGCGGCCGCTCTAGTTTTCCGTACTCTCCAAGA
Pex15REV	CCCCCCGGGAGAACCACCATTCATTGTGGAA
Ylf2FW	AAAGCGGCCGCTTATAATTCATTGCATGATTCTTG
Ylf2REV	CCCCCCGGGGAATCAGCCCACTCTAGGTAAAC
Prk1FW	CCGAGCTC TGATAATTTTAGGTTATGATTGGT
Prk1REV	CGGGATCC GTGGTAAGGCTTCTACTCAACAAG
VirD5#1	CCGCCCGGGGATGACAGGAAAG
VirD5#1-2	CGCCTGCAGGACGGGATCGCTG
VirD5#3-2	CGCCTGCAGCGGCGGAACAAGGAC
VirD5#4-2	CGCCTGCAGTCAGCGTTTAAAC
VirD5#9	CCGCCCGGGGGATAAAAACGAAGCCCC
VirD5#10-2	AAAGCGGCCGCTCAGCGTTTAAACGC
VirD5#21	CCATCGATATGACAGGAAAGTCG
VirD5#21-2	CCCCCCGGGTCAGCGTTTAAAC
VirD5#23	GGACTAGTATGACAGGAAAGTCG
VirD5#23-2	ACGCGTCGACTCAGCGTTTAAAC
VirD5#28	CCGCCCGGGATGACAGGAAAGTCG
VirD5#33	AAAGCGGCCGCAAACAGGAAAGTCGAAAGTTC
VirD5#34	AAAGCGGCCGCAACAGGCTGATGCCTCGTTTG
C58-D5FW	CCGCCCGGGGATGAGACCTTCAGGAAACCCG
C58-D5REV	AAAGCGGCCGCTCAGCGATTGAACGCTTTGT
AB-D5FW	CGCGGATCCACATGAAACCGTCAGGAAACT
AB-D5REV	AAAGCGGCCGCTCATCGGCCGAAGCTCTCG
VirD5#38	GGACTAGTATGACAGGAAAGTCGAAAGTTCAC

VirD5#41	CCGCTCGAGTCAGACGGGATCGCTG
VirD5#63	ACGCGTCGACGGAGATATACCATGGGC
CEN3FW	CAATATGGAAAATCCACAGAAAGCTATTC
CEN3REV	CCACCAGTAAACGTTTCATATATCCATTC
CEN16FW	CATGGTAGTGATCACAAATAGATCACA
CEN16REV	CAACTGAATAATATTTCTATTTTCGGA

Table 3. Plasmids used in this study.

Name	Descriptions	Sources/ references
pAS2.1	High-copy yeast two-hybrid vector with an N-terminal Gal4 binding domain fusion controlled by the ADH promoter.	Clontech
pAS-VirD5	VirD5 (XmaI-PstI) was inserted into pAS2.1.	This study
pAS-VirD5 (1-505)	VirD5 (1-505) (XmaI-PstI) was inserted into pAS2.1.	This study
pAS-VirD5 (1-715)	VirD5 (1-715) (XmaI-PstI) was inserted into pAS2.1.	This study
pAS-VirD5 (716-833)	VirD5 (716-833) (XmaI-PstI) was inserted into pAS2.1.	This study
pGPINTAM-NotI	Binary vector with an tamoxifen inducible promoter.	(Friml *et al*., 2004)
pGPINTAM-Flag-VirD5	VirD5 was inserted into NotI of pGPINTAMNotI.	This study
pMVHIS	High-copy yeast expression plasmid with a GAL1 promoter and a *URA3* marker.	(van Hemert, *et al*., 2003)
pMVHIS-VirD5	VirD5 (XmaI-NotI) was inserted into pMVHis.	This study
pMVHIS-VirD5 (C58)	VirD5 (C58) (XmaI-NotI) was inserted into pMVHis.	This study
pMVHIS-VirD5 (AB2-73)	VirD5 (AB2-73) (BamHI-NotI) was inserted into pMVHis.	This study
pMVHIS-VirD5 (Bo542)	VirD5 (Bo542) (XmaI-NotI) was inserted into pMVHis.	This study
pMVHIS-VirD5 (frame shift)	VirD5 (XmaI-NotI) with frame shift was inserted into pMVHis.	This study
pMVHIS-VirD5 (1-202)	VirD5 (1-202) (XmaI-Xho) was inserted into pMVHis.	This study
pMVHIS-VirD5 (1-505)	VirD5 (1-505) (XmaI-XhoI) was inserted into pMVHis.	This study
pRS315	Single-copy yeast plasmid with a *LEU2* marker.	(Sikorski and Hieter, 1989)
pRS315-Pyk2	*PYK2* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Pex15	*PEX15* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Png1	*PNG1* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Ylf2	*YLF2* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Ptc7	*PTC7* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Tad1	*TAD1* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Prk1	*PRK1* including its own promoter and terminator (SacI-BamHI) was inserted into pRS315.	This study
pRS315-Msb1	*MSB1* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pRS315-Spt4	*SPT4* including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pUG34GFP	Single-copy yeast plasmid for the N-terminal fusion with GFP under the control of the MET25 promoter.	(Sakalis, 2013)

pUG34-GFP-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34GFP.	This study
pUG34-GFP-VirD5NT (1-505)	VirD5NT (1-505) (SpeI-XhoI) was inserted into pUG34GFP.	This study
pUG34-GFP-VirD5CT (521-833)	VirD5CT (521-833) (SpeI-SalI) was inserted into pUG34GFP.	This study
pUG36-CFP	Single-copy yeast plasmid for the N-terminal fusion with CFP under the control of the MET25 promoter.	(Sakalis, 2013)
pUG36-CFP-Ndc10	Ndc10 (BamHI-SalI) was inserted into pUG36CFP .	This study
pUG36-CFP-Spt4	Spt4 (BamHI-SalI) was inserted into pUG36CFP.	This study
pET16H	pBR322 base plasmid with an N-terminal10xHis tag under the control of the T7 promoter.	Novagen
pET16H-VirD5	VirD5 (ClaI-XmaI) was inserted into pET-16H.	This study
pGEX-KG	pMB1 based plasmid with an N-terminal GST tag under the control of the TAC promoter.	(Guan and Dixon, 1991)
pGEX-KG-Spt4	Spt4 (BamHI-SalI) was inserted into pGEX-KG.	This study
pUG34VCn	Single-copy plasmid with an N-terminal fusion with the C-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG34VCn-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34VCN.	This study
pUG35VNc	Single-copy plasmid with an C-terminal fusion with the N-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG35VNc-Spt4	Spt4 (SpeI-SalI) was inserted into pUG35VNC.	This study
pRS315- pGAL1-VirD5NT (1-505)	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5NT (1-505) as template was inserted into SpeI-SalI of pRS315.	This study
pRS425	High-copy yeast plasmid with a *LEU2* marker.	This study
pRS425-pGAL1-VirD5NT (1-505)	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5NT (1-505) as template was inserted into SpeI-SalI of pRS425.	This study
pRS305	Yeast integrative plasmid.	(Gietz and Sugino, 1988)
pRS305-pGAL1-VirD5	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5 as template was inserted into SpeI-SalI of pRS305.	This study

Table 4. Yeast strains used in this study.

Name	Genotypes	Sources/ references
BY4743	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0)	(Brachmann *et al*., 1998)
BY4743:Δspt4	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0/Δspt4:KanMX/ Δspt4:KanMX)	Euroscarf
BY4743:Δpyk2	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δpyk2:KanMX/ Δpyk2:KanMX)	Euroscarf
BY4743:Δpex15	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δpex15:KanMX/Δpex15:KanMX)	Euroscarf
BY4743:Δpng1	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δpng1:KanMX/Δpng1:KanMX)	Euroscarf
BY4743:Δylf2	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δylf2:KanMX/Δylf2:KanMX)	Euroscarf
BY4743:Δptc7	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δptc7:KanMX/Δptc7:KanMX)	Euroscarf
BY4743:Δtad1	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δtad1:KanMX/Δtad1:KanMX)	Euroscarf
BY4743:Δprk1	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δprk1:KanMX/Δprk1:KanMX)	Euroscarf
BY4743:Δmsb1	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/Δmsb1:KanMX/Δmsb1:KanMX)	Euroscarf
BY4743:HTA2-CFP	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15/ura3Δ0/ura3Δ0/HTA2:CFP::KanMX/HTA2:CFP::KanMX/HTA2:CFP::KanMX/HTA2:CFP::KanMX)	(Sakalis, 2013)
BY4743:HTA2:CFP-VirD5	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15ura3Δ0/ura3Δ0/HTA2:CFP::KanMX:pGal1:His-VirD5:LEU2/HTA2:CFP::KanMX:pGal1:His-VirD5:LEU2)	This study
BY4743:VirD5	(MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0/pGal1:His-VirD5::LEU2/ pGal1:His-VirD5::LEU2)	This study
RLY-4029	(MATa,ura3-52,lys2-801;ade2 101;trp1D1;leu2D1; +CFIII (CEN3.L.YFS2.1)URA3;SUP11;leu2D1)	(Chen *et al*., 2012)
RLY-4029:VirD5	(MATa,ura3-52,lys2-801;ade2-101;trp1D1;leu2D1; +CFIII(CEN3.L.YFS2.1)URA3;SUP11;leu2D1;pGal1:His-VirD5::LEU2)	This study
BUY545	(MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 HMRΔI::URA3)	(Dhillon and Kamakaka, 2000)
BUY545(pRS425:VirD5 (1-505))	(MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 HMRΔI::URA3 (pRS425:pGgal1:His-VirD5 (1-505)))	This study
BUY668	(MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 URA3-TELVIIL)	(Dhillon and Kamakaka, 2000)
BUY668(pRS425:VirD5 (1-505))	(MATa ade2-1 his3-11 leu2-3,112 LYS2 trp1-1 ura3-1 ppr1Δ::HIS3 URA3-TELVIIL (pRS425:pGgal1:His-VirD5 (1-505)))	This study
PJ694A	(MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ)	(James, Halladay, and Craig, 1996)

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Chapter 3

The VirD5 protein interferes with mitosis in yeast cells

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Abstract

The kinetochore is a large complex of multiple proteins that assembles at the centromeric region of the chromosome and serves to connect chromosomes to the spindle microtubules during mitosis. It thus plays crucial roles in faithful chromosome segregation. We found that the *Agrobacterium tumefaciens* virulence protein VirD5 is localized via its N-terminal part (NT) at the centromeres/kinetochores and targets the mitotic machinery to effect chromosome mis-segregation. Here, we report that VirD5NT interacts with the essential mitotic regulatory protein Ipl1/Aurora kinase and the outer kinetochore/microtubule-associated protein Dam1. Interaction with the Ipl1/Aurora kinase stimulates its kinase activity. Phosphorylation of substrates such as Dam1 is known to result in the detachment between kinetochore and spindle microtubule. This is necessary for error correction, but increased Ipl1/Aurora kinase activity could lead to spindle checkpoint activation, lagging chromosomes and in the end chromosome mis-segregation. That activation of the Ipl1/Aurora kinase underlies the toxicity of VirD5NT became apparent by artificial boosting of the activity of the specific counteracting phosphatase Glc7, which relieved the toxicity. Apparently, *A. tumefaciens* targets the conserved Aurora kinases to influence cell division and chromosome segregation in the initial phases of infection to advance tumorigenesis in plants.

Introduction

Faithful segregation of duplicated chromosomes over daughter cells during mitosis relies on the attachment of the kinetochore, a large complex of proteins that is made up of more than 60 proteins in yeast to the spindle microtubules (Cheeseman and Desai, 2008; Cho and Harrison, 2012). Structurally the kinetochore is divided into two distinct parts consisting of an inner kinetochore that directly binds to the centromeric DNA of the chromosome, and an outer kinetochore that attaches to the spindle microtubules (Hori *et al*., 2008; Yamagishi *et al*., 2014).

Chromosomes interact with the plus ends of spindle microtubules via their kinetochores and are segregated over daughter cells as the spindle microtubules are depolymerizing and shortening at the onset of anaphase (Kline-smith and Walczak, 2004). In budding yeast, a ten protein Dam1 complex and an Ndc80 complex play essential roles in maintaining the attachment between the kinetochore and the spindle microtubules (Hofmann *et al*., 1998). The Dam1 complex assembles into a 50-nm ring-like structure that embraces the microtubules (Miranda *et al*., 2005). The ring complex remains attached to the assembling tips of spindle microtubules and harness the energy from the depolymerization of microtubules against tension to facilitate chromosome movement during mitosis (Umbreit *et al*., 2014; Westermann *et al*., 2006). Although the Dam1 complex is exclusively present in fungi, a structurally similar Ska1 complex is present in human cells (Schmidt *et al*., 2012). The Dam1 complex binds to the kinetochores through a direct interaction with the Ndc80 complex, an essential outer kinetochore complex consisting of Ndc80, Nuf2, Spc24 and Spc25 (Lampert *et al*., 2013; Wigge and Kilmartin, 2001). The Ndc80 complex forms a rod-shaped heterotetramer that directly binds to the spindle microtubules by its N-terminal calponin homology (CH) domain and interacts with other kinetochore proteins by its C-terminal globular domain (Malvezzi *et al*., 2013).

Chromosomes with erroneous kinetochore-microtubule attachments stimulate the mitotic checkpoint to delay the onset of mitosis until all duplicated chromosomes are bipolarly attached (Sacristan and Kops, 2015). Correction of improper kinetochore-microtubule attachments is achieved by the Aurora B kinase, Ipl1 in budding yeast (Biggins *et al*., 1999; Saurin *et al*., 2011), a component of the Chromosomal Passenger Complex (CPC) further consisting of INCENP/Sli15, Survivin/Bir1 and Borealin/Nbl1 (Carmena *et al*., 2012). Ipl1/Aurora B kinase is thought to destabilize erroneous attachments via phosphorylation of key proteins including Ndc80/Hec1 and Dam1/Ska1 that are involved directly in the kinetochore-microtubule attachment (DeLuca, Lens, and DeLuca, 2011; Schmidt *et al*., 2012; Umbreit *et al*., 2014). Therefore, a reduction in the activity of Aurora B/Ipl1 leads to chromosome mis-segregation and aneuploidy. Overexpression of Aurora B/Ipl1, however, also leads to defective chromosome segregation as the repeated disruption of kinetochore-microtubule attachments not only activates the Spindle Assembly Checkpoint (SAC), but in the end generates lagging chromosomes and aneuploid cells (Etemad, Kuijt, and Kops, 2015; Muñoz-Barrera and Monje-Casas, 2014).

In our previous study, we found that the *A. tumefaciens* virulence protein VirD5 affects mitosis and causes chromosome mis-segregation in budding yeast. Here investigated further the molecular basis of the toxicity of VirD5 and found evidence that VirD5 directly interacts

with the conserved Ipl1/Aurora kinase and that by enhancing its kinase activities causes chromosome mis-segregation and aneuploidy.

Results

VirD5 directly binds to yeast centromeric DNA

The centromere is the chromosomal locus that is required for the assembly of the kinetochore and thus for the delivery of one copy of each chromosome into the daughter cells during mitosis. Centromeric DNA is extremely diverse among eukaryotes, ranging from the simple ~125bp centromere of *S. cerevisiae* to the highly repetitive α-satellite regions of vertebrates (Burrack and Berman, 2012; Carroll and Straight, 2006). It was shown that VirD5 contains several DNA binding motifs (Schrammeijer *et al*., 2000) and is localized at the centromeres/kinetochores in yeast nuclei (**Chapter 2**), and therefore, we wondered whether VirD5 could directly bind to the centromeric DNA of yeast. To test this, we carried out an Electrophoretic Mobility Shift Assay (EMSA) using the centromeric DNA from chromosome 3 (CEN3) and chromosome 16 (CEN16) as probes. Primers labeled by Cy5 at their 5'ends (**Table 1**) were used to amplify yeast CEN3 and CEN16 fragments. His-tagged VirD5 expressed and purified from *E.coli* was incubated either with labeled CEN3 or CEN16 *in vitro* for 30 minutes at room temperature, whereafter the DNA-protein mixtures were separated in a 2% agarose gel. As can be seen in **Figure 1A** and **B**, lanes 2, two shifted bands were clearly observed after VirD5 had been incubated with these centromeric DNA probes, whereas no extra band was visible in control experiments lacking VirD5 (**Figure 1A** and **B**, lanes 1). To confirm the specificity of the binding activity, non-labelled CEN3 or CEN16 were used as the competitive DNA. The presence of a 100-fold excess of competitor fragments completely eliminated the band shifts (**Figure 1A** and **B**, lanes 3). These data indicate that VirD5 can specifically bind to yeast centromeric DNA.

In budding yeast, the centromeric DNA consists of three regions named CDEI, CDEII and CDEIII, each of which is bound by distinct proteins (Hemmerich *et al*., 2000; Westermann, Drubin, and Barnes, 2007). CDEI, an 8 bp conserved DNA region (ATCACGTG), is recognized and bound by centromere binding factor 1 (CBF1) (Hemmerich *et al*., 2000); CDEII, a 78-86 bp core element consisting of AT-rich DNA, is specifically wrapped by nucleosomes with the histone 3 variant Cse4 (Camahort *et al*., 2007); CDEIII contains 25 bp that recruits the centromere binding factor 3 (CBF3) complex as a platform for inner kinetochore assembly (Lechner and Carbon, 1991). To determine which part of the centromeric DNA could be bound by VirD5, we digested the double Cy5 end-labelled CEN16 DNA with DraI (**Figure 1C**) to obtain two single-end labelled small fragments, which were subsequently separately incubated with His-tagged VirD5 *in vitro*. As shown in **Figure 1D**, VirD5 exclusively bound to the left region of CEN16 which contains CDEI and a part of CDEII.

VirD5 interacts with kinetochore-associated proteins

Kinetochores are large protein complexes that assemble only at centromeres and interact with spindle microtubules to establish bipolar attachment of paired sister chromatids during mitosis. Structurally, kinetochores consist of an inner kinetochore, which is bound directly to the centromere and an outer kinetochore, which mediates spindle microtubule attachment

(Burrack and Berman, 2012; Hemmerich *et al*., 2000). In budding yeast, each kinetochore is attached to only a single microtubule. Recently more than 60 yeast kinetochore proteins have been identified, many of which are conserved among eukaryotes (Biggins, 2013). To test whether VirD5 interacts with proteins which are associated with the kinetochore, we performed Bimolecular Fluorescent Complementation (BIFC) experiments (Kerppola, 2008). Candidate proteins (**Table 2**) from the inner and outer kinetochore were fused with the N-terminal part of YFP (VN173), and introduced into yeast cells together with VirD5 fused with the C-terminal part of YFP (VC173). The outer kinetochore/microtubule-associated protein Dam1 and the essential mitosis regulatory Ipl1/Aurora kinase displayed a very strong BIFC YFP signal with VirD5 (**Figure 2A**, a-c, g-i), while the fusion of Dam1or Ipl1 together with the unfused complementary part did not give a YFP signal (**Figure 2A**, d-f and j-l). No interactions with any of the other tested kinetochore proteins were observed (**Table 2**).

To confirm the interactions, an *in vitro* pull-down assay was carried out: Dam1 or Ipl1 protein fused to the GST tag was expressed in *E.coli* and bound to the Glutathione HiCap Matrix as the bait. The beads were incubated separately with His-tagged VirD5 purified from *E.coli*, for 2 hours at room temperature in binding buffer containing 0.1% Triton-100. After 3 times washing, the protein mixtures were separated on a SDS-PAGE gel. The presence of His-tagged VirD5 was detected by Western blot using anti-His antibody. As can be seen in **Figure 2C**, VirD5 physically interacted with GST-tagged Dam1 and GST-tagged Ipl1, but not with empty GST.

The interactions of VirD5 with Dam1 and Ipl1 requires Spt4

If interference with the functioning of the kinetochore is causing the toxicity of VirD5 its presence at the kinetochore would be imperative for toxicity. Deletion of the *spt4* gene altered the sublocation of VirD5 as the punctate foci in the nucleus disappeared (**Chapter 2**). We were therefore interested to find out whether the interactions of VirD5 with Dam1 and Ipl1 rely on Spt4 in the cell. To this end we introduced the BIFC vector 34VCn-VirD5 either with 35VNc-Dam1 or 35VNc-Ipl1 into the *spt4* deletion mutant to determine the BIFC signal. As shown in **Figure 2D**, no YFP signal was observed in *spt4* mutant cells expressing VirD5 together with either Dam1 or Ipl1, demonstrating that the interaction with Dam1 and Ipl1 is dependent on Spt4. The weak interactions of VirD5 with Ipl1 and Dam1 *in vitro* suggests that *E.coli* might have an Spt4-like elongation factor or charged amino acids could have provided an interaction site *in vitro* at high protein concentration.

VirD5 disturbs spindle microtubule elongation and chromosome segregation over the daughter cells

The Dam1 protein forms rings encircling the microtubules, that translate the force generated by depolymerization of the microtubules into movement of chromosomes attached via the kinetochore (Franck *et al*., 2007). In order to visualize the microtubule, we labelled tubulin with GFP. The spindle microtubule in wild type cells elongated into both mother and daughter cells during mitosis. In contrast, in the presence of VirD5, more than 80% of anaphase cells had short spindle microtubules that did not manage to enter the daughter cell (**Figure 3A**). To further confirm these spindle elongation defects caused by VirD5, we endogenously labeled the Dam1 protein with a 3xGFP tag at its C-terminus. Dividing cells

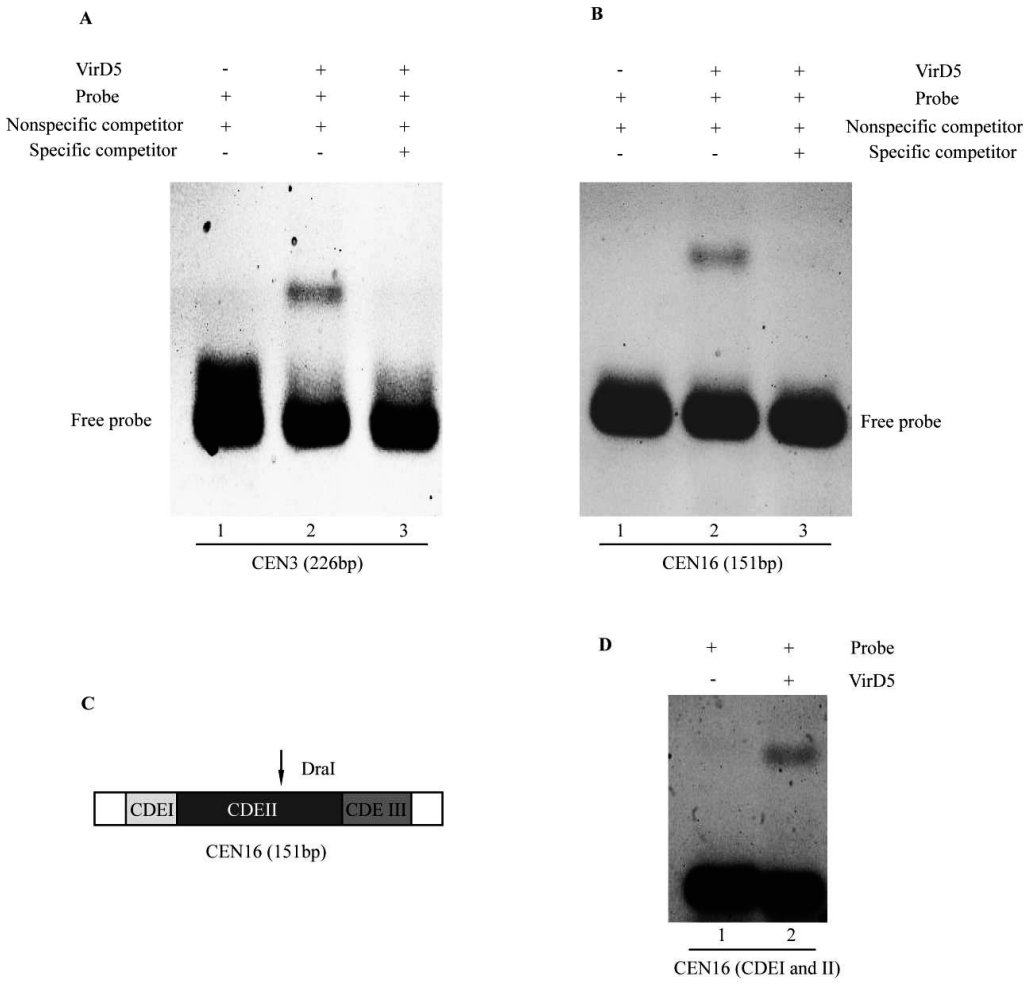


Figure 1. VirD5 binds to yeast centromeric DNA. (A-B) EMSA were performed using yeast centromeric DNA from chromosomes 3 (A) and 16 (B) as probes. His-tagged VirD5 purified from *E.coli* was incubated with probes. Non-labelled CEN3 or CEN16 was added as the specific competitor. (C) Representation of the DraI restriction site within CDEII of CEN16. (D) The left region of CEN16 containing CDEI and a part of CDEII was used for EMSA with or without the addition of His-tagged VirD5. All experiments were repeated three times.

showed two bright dots, one in the mother and the other that had moved into the daughter cell (**Figure 3B**, upper panel). In the presence of VirD5 both dots representing the position in the cells where the chromosomes are located were still close together confirming the spindle elongation defect. The two dots were either distributed over mother and daughter cell (56%) as in the absence of VirD5 or lagging in the middle of the mother cell (42%) (**Figure 3B**, middle and lower panel). These results demonstrate that VirD5 interferes with spindle microtubule elongation and entrance of the spindle into daughter cells.

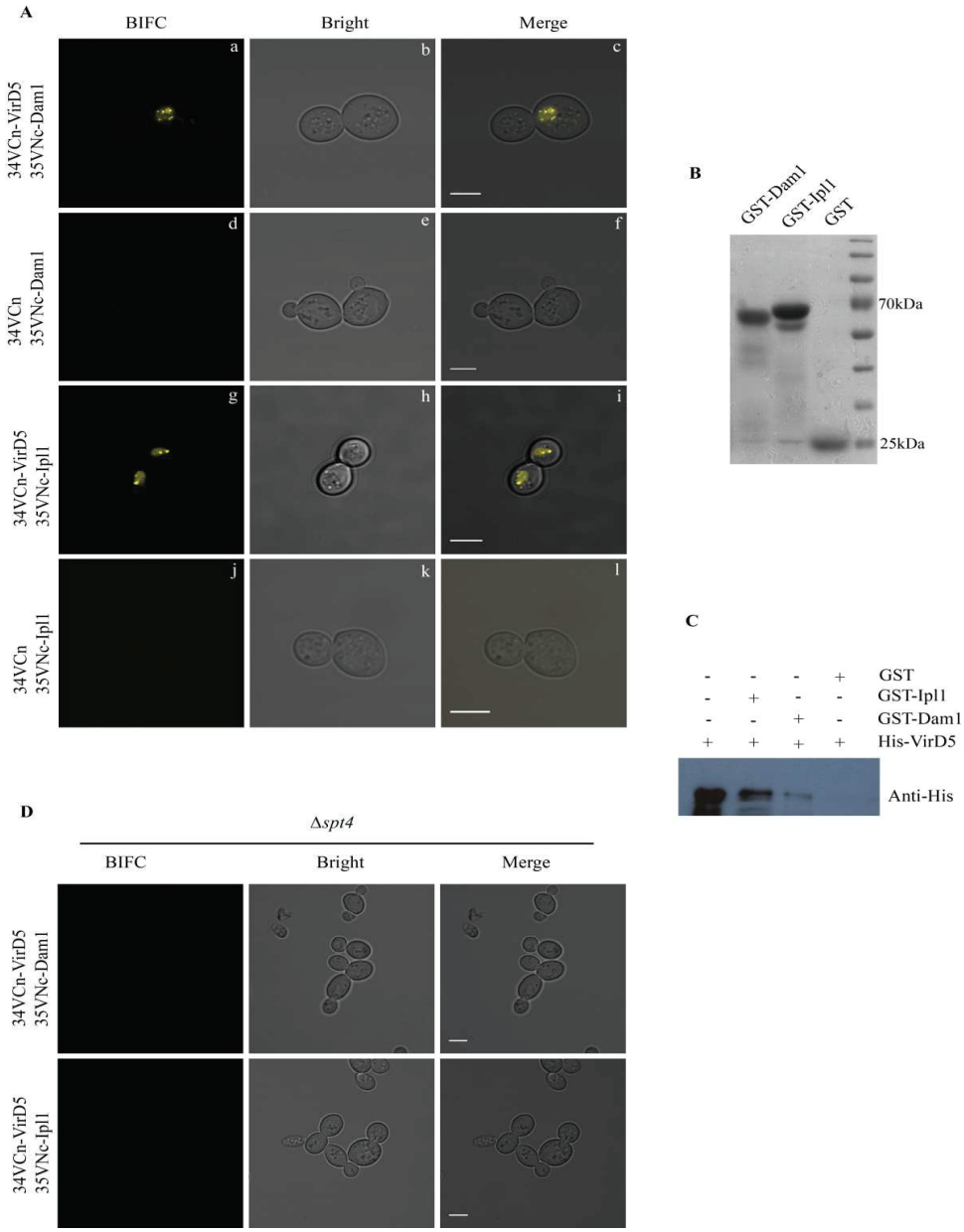


Figure 2. VirD5 physically interacts with kinetochore-associated proteins. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP (VC173) fused with the N-terminus of testing proteins. 35VNc, the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. Scale bar, 5 μm. (B) GST or GST-tagged proteins were expressed and purified from *E. coli*. (C) His-tagged VirD5 purified from *E. coli* was incubated with either empty GST or GST-Ipl1 or GST-Dam1, and after washing steps the presence of His-VirD5 was detected by anti-His antibody. (D) BIFC vector 34VCn-VirD5 either with 35VNc-Dam1 or 35VNc-Ipl1 were transformed into Δ*spt4* cells for BIFC detection. Scale bar, 5 μm.

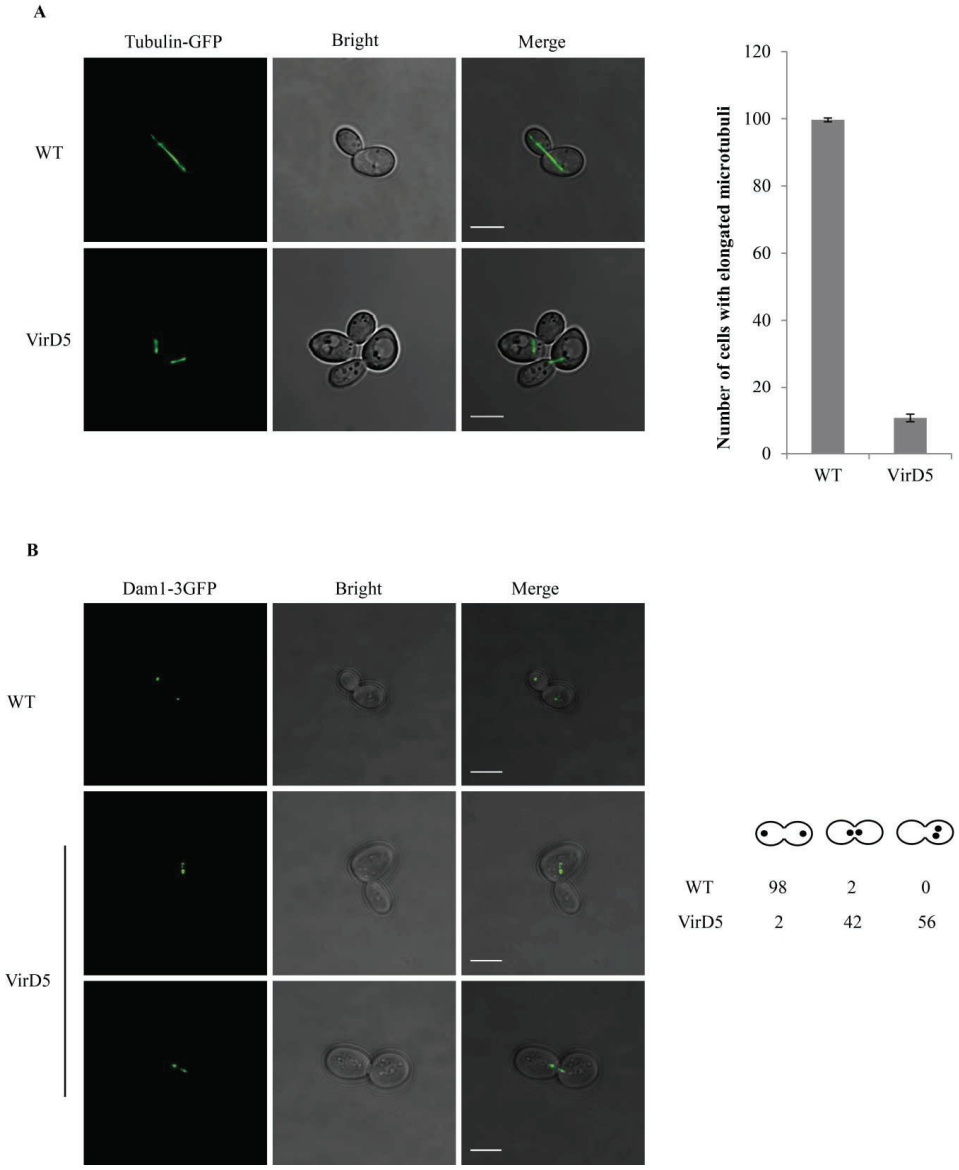


Figure 3. VirD5 disturbs the separation of sister kinetochores and spindle microtubule elongation during anaphase. (A) Tubulin-GFP marked MAS101 cells with or without the insertion of *virD5* driven by the GAL1 promoter were grown in rich medium containing glucose first and shifted to rich medium containing 2% galactose. 100 cells were counted per experiment. Scale bar, 5 μm. Error bars represent the mean ± SD from three independent experiments. (B) The kinetochore/microtubule-associated Dam1-3xGFP marked yeast cells with or without the integration of *virD5* under the control of the GAL1 promoter were grown in rich medium containing glucose first and shifted to rich medium containing 2% galactose. GFP dots were visualized under the confocal microscope. Scale bar, 5 μm. All experiments were repeated at least three times.

In the presence of VirD5 the Ipl1 kinase has an altered subcellular localization in anaphase

The spindle microtubules attach to the kinetochores and pull to separate the chromosomes destined for mother and daughter cells during mitosis. For faithful chromosome segregation, sister kinetochores must attach to spindle microtubules emanating from the two opposite poles (Foley and Kapoor, 2013; Tanaka, 2005). Incorrect attachments provoke the spindle assembly checkpoint (SAC), which arrests cells in metaphase until incorrect chromosome-microtubule attachments have been corrected (Sacristan and Kops, 2015). In yeast, the essential Aurora kinase called Ipl1, displays a dynamic subcellular localization during the cell cycle being present at the centromeres from interphase until metaphase, but at the spindle midzone during anaphase. It phosphorylates several kinetochore and microtubule-associated proteins leading to the detachment of incorrect chromosome-microtubule attachments (Biggins *et al*., 1999; Cheeseman *et al*., 2006; Keating *et al*., 2009). Earlier work in this chapter showed that VirD5 interacted with the Ipl1 kinase and is present at the centromeres/kinetochores. We therefore wondered whether the presence of VirD5 may alter the subcellular localization of Ipl1. As shown in **Figure 4A**, in wild type cells Ipl1 accumulated at the spindle midzone between the spindle pole bodies in anaphase as expected. In contrast, in cells expressing VirD5, a diffuse nuclear signal was seen, pointing to a defect in maintaining Ipl1 at the spindle midzone in anaphase.

VirD5 stimulates the kinase activity of Ipl1/Aurora on Dam1

Ipl1/Aurora kinase plays crucial roles in sensing and correcting the erroneous kinetochore-spindle microtubule attachments by phosphorylating key substrates involved in the kinetochore-spindle binding. Both loss and overexpression of the Ipl1/Aurora kinases leads to massive chromosome mis-segregation and aneuploidy in yeast cells (Chan and Botstein, 1993; Muñoz-barrera and Monje-casas, 2014*)*. Earlier work in this chapter showed that VirD5 interacts with Ipl1 and disturbs its spindle midzone localization in anaphase and thus may influence its kinase activities. In order to find out whether VirD5 may affect the kinase activity of Ipl1, we carried out an *in vitro* kinase assay using the well-known microtubule binding protein Dam1 as the substrate (Kang *et al*., 2001). GST-Dam1 expressed and purified from *E.coli*, was mixed with His-tagged Ipl1 in the presence or absence of His-tagged VirD5 in a phosphorylation buffer containing [γ-^{32}P] ATP; subsequently, the protein mixtures were separated on a SDS-PAGE gel and analyzed by autoradiography. We observed a stronger P^{32} signal on the Dam1 substrate that had been incubated with Ipl1/Aurora kinase in the presence of VirD5 (**Figure 4B**), than in control experiments in the absence of VirD5, which itself showed no kinase activity on Dam1. This suggests that VirD5 may stimulate the kinase activity of Ipl1/Aurora kinase during their interaction in yeast.

The N-terminus of VirD5 interacts with Ipl1 and Dam1

In order to find out which part of VirD5 could bind to the Ipl1 kinase and the outer kinetochore/microtubule associated protein Dam1, we performed a BIFC assay using serveral truncations of VirD5 and found that the N-terminal 505 amino acids of VirD5 (VirD5NT) gave strong interaction signals with both Ipl1 and Dam1 (**Figure 5A**). In our previous study we found that this same VirD5NT was responsible for localization at the centromeres/kinetochores and led to growth inhibition in yeast cells (**Chapter 2**). This

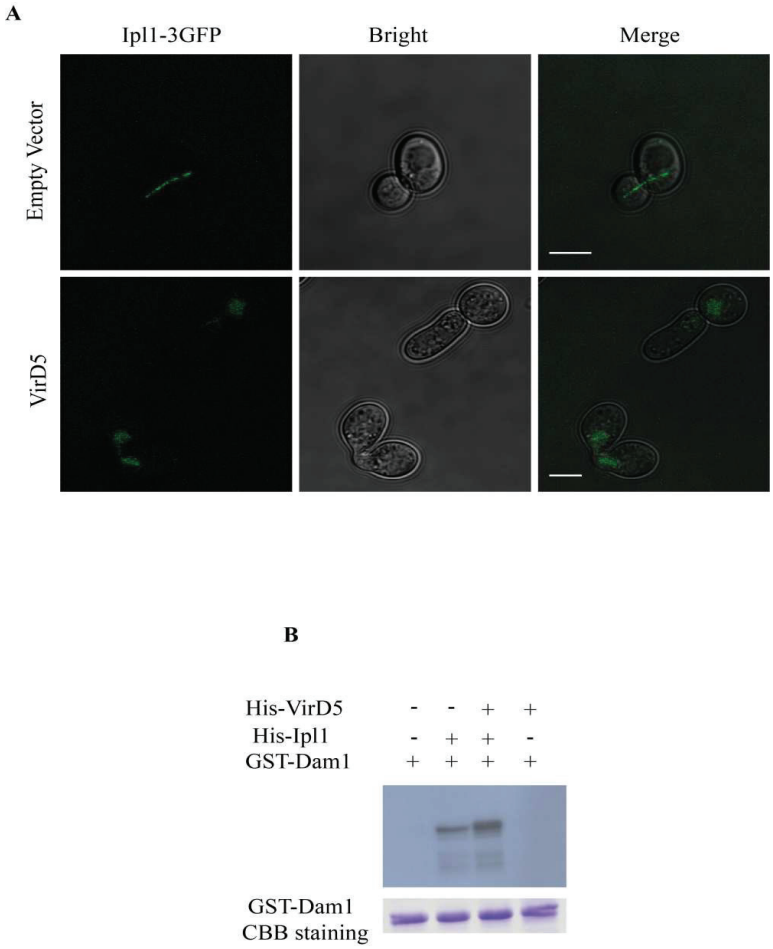


Figure 4. VirD5 stimulates the kinase activity of Ipl1. (A) Ipl1-3xGFP marked cells (YB3039) were transformed with either empty plasmid or plasmid containing *virD5* under the control of the MET25 promoter. After shifting to methionine free medium, GFP signal was observed by the confocal microscope. Scale bar, 5 μm. 100 cells per experiment were observed. (B) GST-tagged Dam1 purified from *E. coli* was incubated with His-tagged Ipl1 with or without His-tagged VirD5 protein in kinase buffer containing [γ-^{32}P] ATP. Coomassie Brilliant Blue (CBB) showed that equal amounts of GST-Dam1 were used for the kinase assay.

evoked our interest in the N-terminal part of VirD5. Further protein sequence alignment showed that VirD5NT contains 6 degenerate repeats each of which consists of around 40 amino acids (**Figure 5B**). To determine whether these repeats are all essential for the toxicity, VirD5NT and VirD5 derivatives with a deletion of each individual repeat were expressed under the control of the GAL1 promoter in yeast BY4743 cells. After induction on galacose containing medium , we found that the deletion of repeat 5 had led to a robust loss of toxicity of both VirD5NT (**Figure 5C**) and VirD5 (data not shown). In order to find out why toxicity of VirD5 lacking repeat 5 was diminished, we analyzed whether this protein still was localized at the centromers and whether it still could interact with Ipl1.

In a BIFC experiment we found that the VirD5NTΔR5 protein in contrast to VirD5 no longer gave a fluorescence signal with Ipl1 and only a weak signal with Dam1 (**Figure 6A**). This indicates that VirD5NTΔR5 no longer interacts with Ipl1 explaining its loss of toxicity. In order to find out whether this loss of interaction is due to an inability to localize at the centromeres/kinetochores we next determined the subcellular localization of VirD5NT and VirD5NTΔR5. We found that both proteins were still targeted to the centromeres/kinetochores in the nucleus (**Figure 6B**), although the numbers of fluorescent foci (1-4) were lower than seen with full length VirD5 (**Chapter 2**), indicating that targeting to the centromeres/kinetochores by VirD5NT is independent of repeat 5. Apparently, repeat 5 is important for a strong interaction with both Ipl1 and Dam1.

The toxicity of VirD5NT is suppressed by the Glc7 phosphatase and its regulatory proteins

It has been found that protein phosphatase I (Glc7 in budding yeast) can oppose the kinase activity of Ipl1/Aurora kinase by dephosphorylating the same substrates to tightly regulate the cell cycle during mitosis (Francisco, Wang, and Chan, 1994; Hsu *et al*., 2000; Pinsky, *et al*., 2006). The Glc7 activity is regulated by several regulatory (activating) subunits such as Glc8, Gip3 and Gip4 (Nigavekar *et al*., 2002; Pinsky *et al*., 2006). If the toxicity of VirD5 is indeed based on the stimulation of the kinase activity of Ipl1, overexpression of the opposing phosphatases should be able to rescue cells from toxicity. To test this, we used a truncation of VirD5 consisting of the N-terminal 505 amino acids (VirD5NT) which was shown to interact with the Ipl1 kinase (**Figure 5A**) to carry out a suppressor assay. As shown in **Figure 6C**, VirD5NT inhibited the growth of yeast cells, but this growth inhibition was indeed suppressed by overexpression of Glc7 or any of its three activating regulatory proteins. This indicates that indeed the increased kinase activity of Ipl1 underlies the toxicity of VirD5NT.

VirD5 induces chromosome mis-segregation

In our previous study we found that VirD5 induces aneuploidy which is a consequence of chromosome mis-segregation (**Chapter 2**). To confirm chromosome mis-segregation caused by VirD5 directly, we integrated *virD5* driven by the GAL1 promoter into the haploid yeast strain Y716 (Meyer *et al*., 2013). This strain expresses a GFP-LacI fusion protein that binds to a 256 tandem repeat *lac*O operator array integrated into chromosome I of this yeast strain, thus marking chromosome I with a green fluorescent bright dot inside the nucleus that can be seen under the microscope. In the presence of galactose, cells lacking VirD5 showed equal chromosome I segregation (**Figure 7**, a-c), whereas approximately 97% of the cells expressing VirD5 displayed an erroneous distribution of chromosome I over the daughter cells (**Figure 7**, d-l). We found three classes of mis-segregation: the major class represents mother cells with two chromosomes I, and daughter cells lacking chromosome I (**Figure 7**, g-i), the second class comprises mother cells with chromosome I, and daughters lacking chromosome I (**Figure 7**, d-f), and the last class of cells represents pairs of cells with in total more than two bright GFP dots (**Figure 7**, j-l). VirD5NT was shown to affect mitosis and inhibit yeast growth (**Chapter 2**).

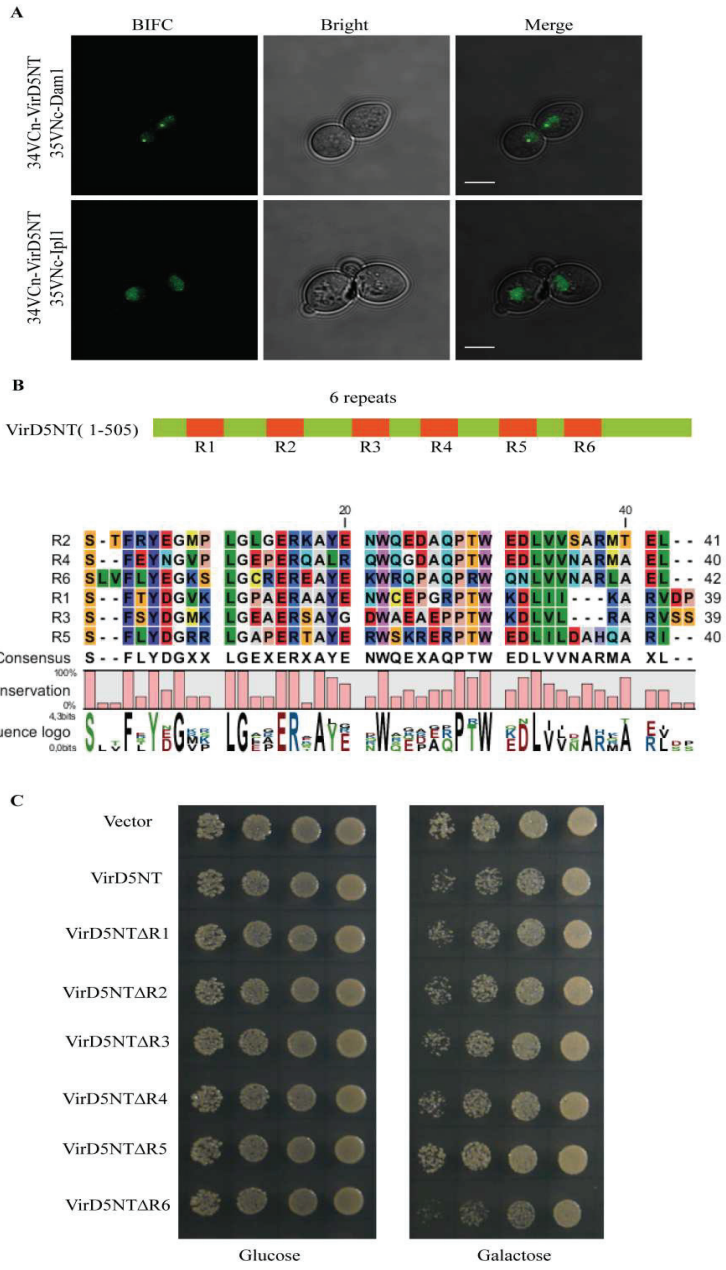


Figure 5. The N-terminus of VirD5 interacts with Ipl1 and Dam1. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP fragment (VC173) fused with the N-terminus of testing proteins. 35VNc, the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein. Scale bar, 5 μm. (B) Sequence alignment of the N-terminal repeats of VirD5. (C) Empty vector or derivatives expressing either VirD5NT or VirD5NT lacking an individual repeat driven by the GAL1 promoter were transformed into diploid yeast cells (BY4743). Transformants were serially diluted and spotted onto selection medium containing either glucose or galactose.

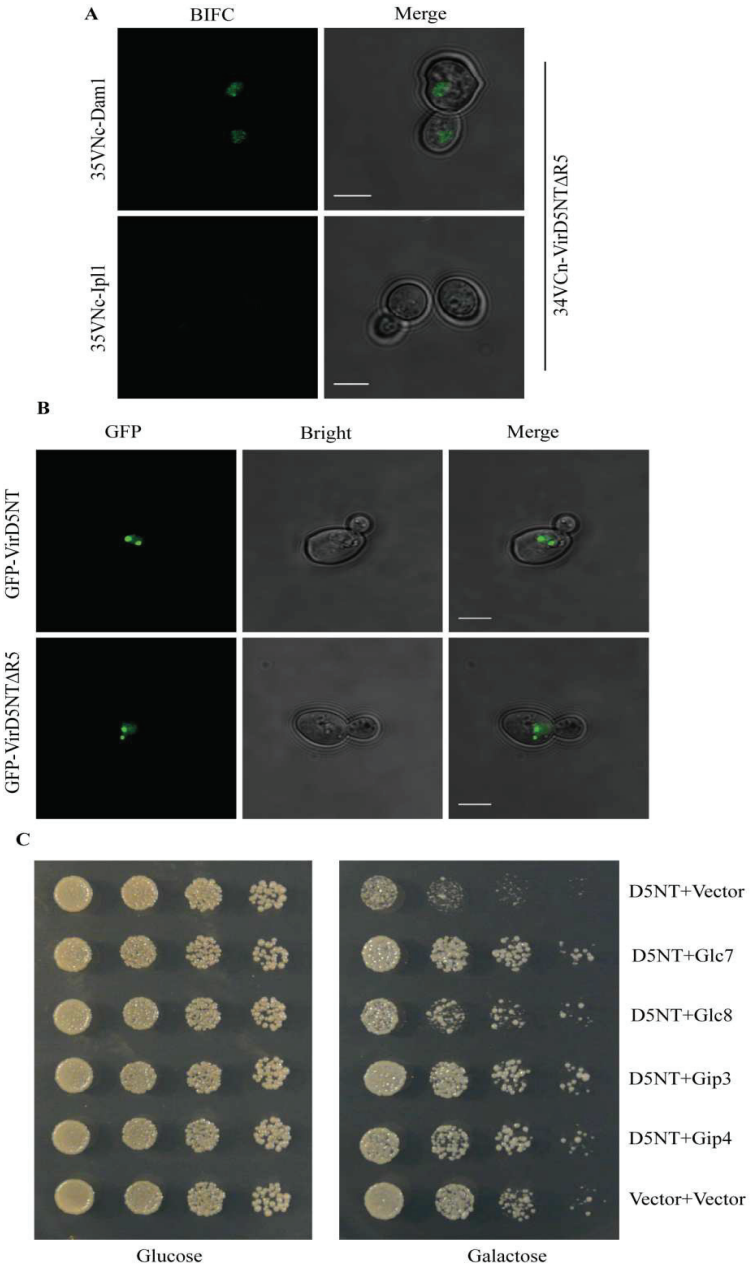
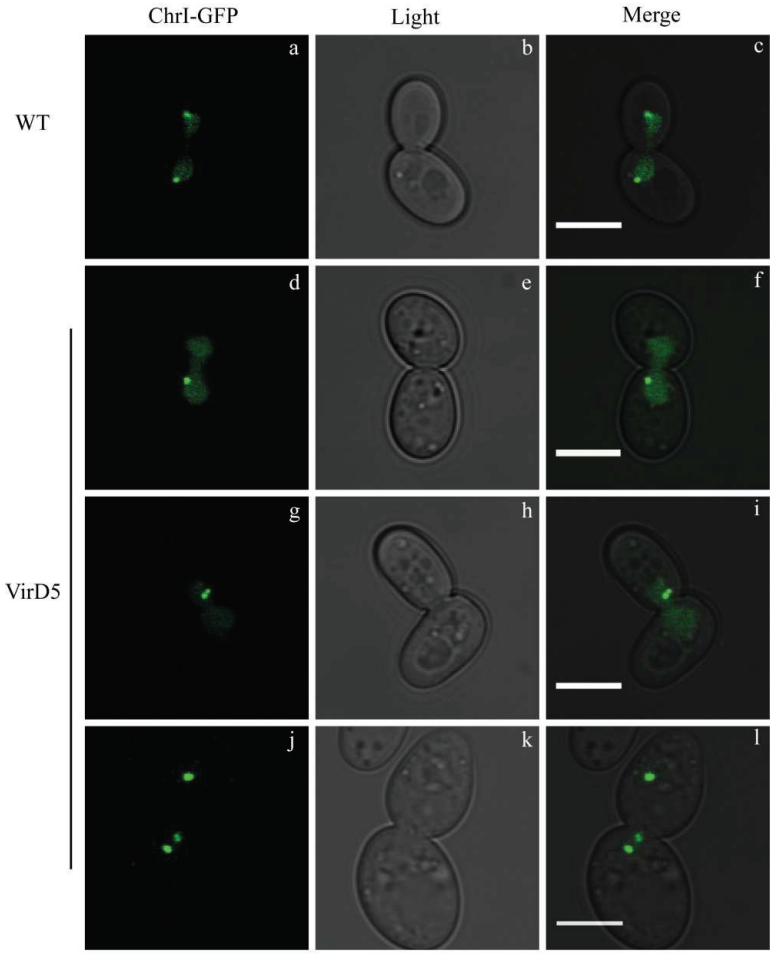


Figure 6. The toxicity of VirD5NT is suppressed by different phosphatases. (A) Yeast cells were transformed with BIFC vectors. Scale bar, 5 μm. (B) Yeast cells were transformed with plasmid encoding either GFP-VirD5NT or GFP-VirD5NTΔR5. Scale bar, 5 μm. (C) Plasmid containing *virD5NT* driven by the GAL1 promoter was cotransformed into haploid BY4741 cells either with a high-copy empty plasmid or the same plasmid expressing phosphatase Glc7 or its activators from their own promoters and terminators. Transformants were serially diluted and spotted onto selection medium containing either glucose or galactose. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein.



WT	100	0	0	0
VirD5	3	42	46	9
VirD5NT	72	10	15	3

Figure 7. VirD5 disturbs chromosome segregation. The yeast strain Y716 contains a 256 repeat LacO array in Chromosome I and expresses GFP-LacI. Binding of GFP-LacI to the LacO array allows the visualization of a GFP dot. Wild type cells or cells with integration of either *virD5* or *virD5NT-3xNLS* under the control of the GAL1 promoter were cultured in rich medium containing glucose first, and were shifted to galactose containing rich medium. 100 mitotic cells from each experiment were observed. Scale bar, 5 μm.

We thus wondered whether VirD5NT was responsible for chromosome mis-segregation. To this end we examined the distribution of chromosome I dots in cells (Y716) expressing VirD5NT and found that approximately 30% of cells expressing VirD5NT displayed chromosome mis-segregation (**Figure 7**).These results confirm that VirD5 disturbs accurate chromosome segregation and consequently results in the generation of aneuploidy. Moreover, they indicate that VirD5NT is capable of inducing chromosome mis-segregation, but that the full length protein is much more effective.

VirD5 induces DNA damage

Previously we found that chromosomes isolated from cells expressing VirD5 cells were prone to breakage and fragmentation as visualized as a massive loss of clear chromosome bands on CHEF gels (**Chapter 2**). To find more direct evidence for the presence of chromosomal DNA breaks in cells expressing VirD5, we expressed VirD5 under the control of the GAL1 promoter in Rad52-GFP marked yeast cells. Visualization of foci of Rad52, the key protein involved in homologous DNA repair, allows to count the number of sites where DNA double-stranded breaks are being repaired. Using this strain we found that over 90% of the cells expressing VirD5 displayed green dots representing DNA repair foci in the nucleus, while only very few cells lacking VirD5 (~5%) showed a single DNA repair focus (**Figure 8A** and **B**).

We found that VirD5NT increased the frequency of chromosome mis-segregation (**Figure 7**), but to a lesser extent than the full length VirD5 protein. In order to find out whether VirD5NT would be sufficient for the induction of DNA breaks, we counted the number of Rad52-GFP foci in cells with a chromosomally integrated construct encoding VirD5NT. As can be seen in **Figure 8B**, cells expressing VirD5NT showed a more than two-fold higher number of DNA repair foci compared with wild type cells. We next determined whether this increased DNA damage caused by VirD5NT could be strong enough to trigger the massive chromosomal fragmentation seen previously with chromosomal preparations from cells expressing VirD5. To this end chromosomes from yeast cells with a chromosomally integrated plasmid encoding either GFP or GFP-VirD5NT under the control of the MET25 promoter, or from cells with a high-copy empty plasmid or plasmid containing *virD5NT* driven by the GAL1 promoter, were isolated and separated in a CHEF gel. The N-terminal part of VirD5 did not generate the massive chromosomal fragmentation seen after expression of the full length protein (**Figure 8C**), although the presence of VirD5NT increased the numbers of Rad52-GFP foci in yeast cells (**Figure 8B**). These results strongly suggest that VirD5 causes DNA breaks, but this property requires both the N-terminal and the C-terminal parts of VirD5.

Discussion

In this study, we have shown that VirD5 directly binds to the centromeric DNA (**Figure 1**) which confirmed our previous bioinformatics prediction showing that VirD5 contains several DNA binding motifs (Schrammeijer *et al.*, 2001). Whether this DNA binding activity has any biological meaning is unclear at the moment, but in any case is not strong enough to bring VirD5 to the centromeres as in the absence of Spt4 the protein is no longer localized at the centromeres (**Chapter 2**).

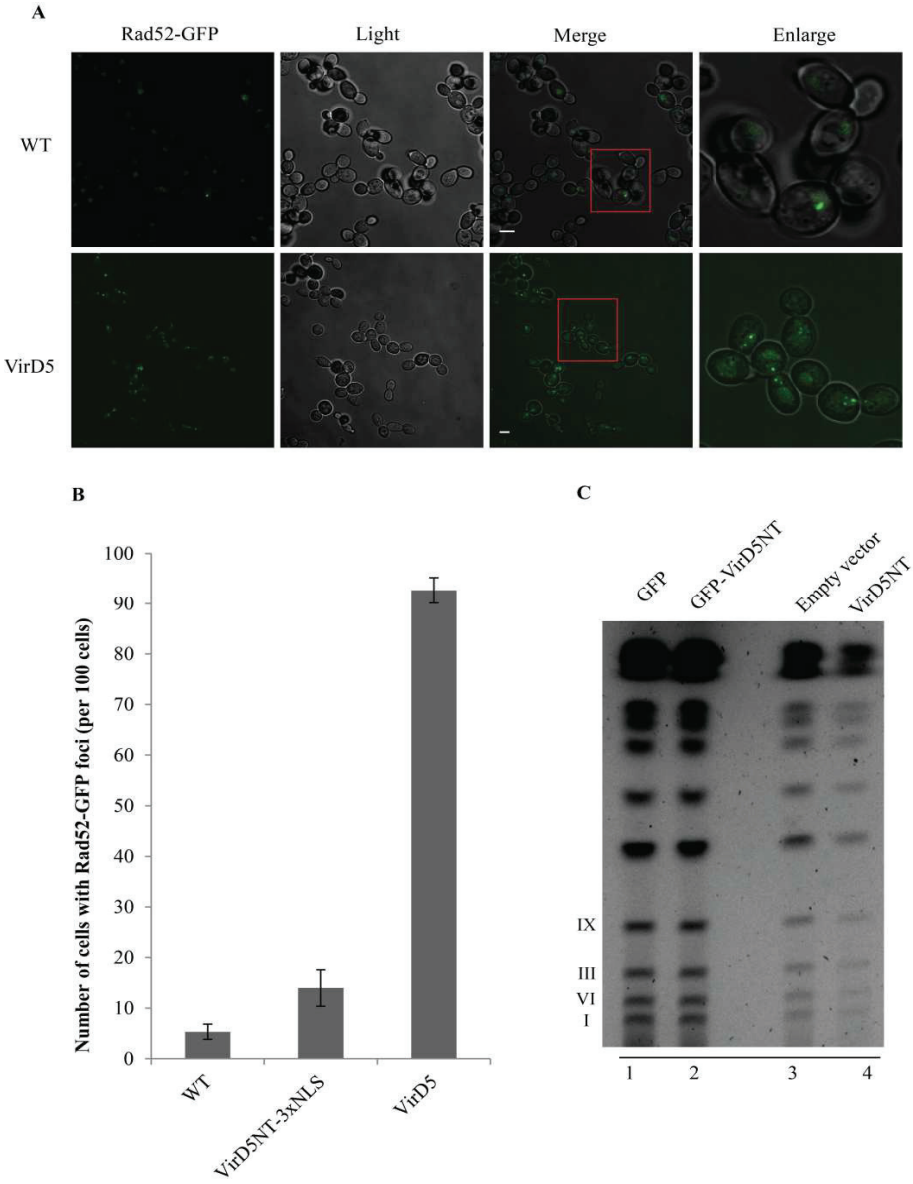


Figure 8. VirD5 causes massive DNA damage. (A-B) A Rad52-GFP marked strain with or without the integration of either the *virD5* or *virD5NT-3xNLS* gene driven by the GAL1 promoter was cultured in rich medium containing glucose, followed by a shift to rich medium containing galactose. The DNA repair foci were observed and counted under the confocal microscope. Scale bar, 5 μm. Error bars represent the mean ± SD from three independent experiments. (C) Chromosomes from yeast (BY4743-H2A-CFP) with a chromosomally integrated construct encoding either GFP (1) or GFP-VirD5NT (2), or from BY4743 transformed either with a high-copy empty plasmid (3) or a plasmid encoding VirD5NT driven by the GAL1 promoter (4), were isolated and separated in a CHEF gel. VirD5NT, the N-terminal 505 amino acids of the VirD5 protein.

The presence of VirD5 did not disturb the co-localization pattern of the centromeres/kinetochores in the cell as both in absence and in presence of VirD5 the kinetochore protein Dam1 showed the same single dots in the nucleus as visualized after GFP fusion (**Figure 3B**). However, VirD5 interfered with spindle elongation, positioning at the bud and entrance in the daughter cell in anaphase (**Figure 3A**), thus preventing the faithful sister-chromatid separation over the daughter cells during mitosis (**Figure 3B**). Also the fast elongation of the microtubules seen in the absence was no longer seen in the presence of VirD5 indicating that cells with VirD5 are arrested by the spindle attachment checkpoint (SAC) or otherwise suffer from an inhibitory effect on spindle microtubule elongation. The spindle microtubules only elongate after proper amphitelic attachment of all the kinetochores and then separate the chromatids from each other by pulling them to the opposite spindle poles. Erroneous attachment of any of the kinetochores activates the Ipl1/Aurora kinase, which phosphorylates outer kinetochore proteins of the Ndc80 complex and proteins of the Dam1 complex associated with the microtubules, which leads to detachment (Cheeseman *et al*., 2002; DeLuca *et al*., 2011; Saurin *et al*., 2011). If any of the kinetochores is not attached to a microtubule the SAC is activated and only inactivated after proper attachment of all the kinetochores is realized (Sacristan and Kops, 2015). We found that VirD5, when present through interaction with Spt4 at the kinetochore, could interact with both the Dam1 complex and Ipl1/Aurora kinase (**Figure 2A** and **C**). The yeast Dam1 complex encircles the plus ends of microtubules (Miranda *et al*., 2005), and is essential for binding of the microtubules to the kinetochores through a direct interaction with the outer kinetochore protein Ndc80 (Lampert *et al*., 2013; Tien *et al*., 2010). The human Ska1 protein complex was recently identified as having a similar structure and function as the Dam1 complex in facilitating microtubule movement (Welburn *et al*., 2009). Irregular expression of Aurora kinases causes chromosome mis-segregation and tumor formation (Demidov *et al*., 2014; Katayama *et al*., 2003; Vader and Lens, 2008).

We found that VirD5 could interact *in vivo* at the kinetochores with the Ipl1/Aurora kinase (**Figure 2A** and **C**) and could stimulate its kinase activity and phosphorylation of the substrate Dam1 *in vitro* (**Figure 4B**). This explains why VirD5 and VirD5NT which interacts with Ipl1 and Dam1 (**Figure 5A**) cause chromosome mis-segregation and aneuploidy formation (**Figure 7**). In line with this, the toxicity of VirD5NT could be suppressed by overexpression of the Glc7 phosphatase or its activation proteins, which dampens the hyperactivity of Ipl1 due to the presence of VirD5NT (**Figure 6C**). The Glc7 phosphatase is involved in dephosphorylation of proteins phosphorylated by the Ipl1/Aurora kinase (Francisco, Wang, and Chan, 1994; Hsu *et al*., 2000; Pinsky, *et al*., 2006). We have also observed that VirD5 physically interacts with other Aurora kinases from both human and plant cells (**Chapter 4** and **5**), and found the same disturbed chromosome segregation in those cells.

It has been shown that chromosome mis-segregation can lead to DNA breaks (Janssen *et al*., 2011). In the presence of VirD5NT cells mis-segregated their chromosomes (**Figure 7**) and had more DNA damage than control cells (**Figure 8B**). This DNA damage associated with the presence of VirD5NT may thus be due to chromosome mis-segregation. The full length VirD5 protein is much more toxic than VirD5NT and this may be due to the fact that the presence of full length VirD5 generates massive DNA damage and chromosomal

fragmentation (**Figure 8C**, **Chapter 2**). This indicates that the C-terminal part of VirD5 (VirD5CT) may reinforce the biological activities of the N-terminal part or contribute other independent toxic (DNA damaging) activities. Indeed we have found that the C-terminal part of VirD5 on its own also triggers massive DNA damage (our unpublished results). Both of these biological activities of VirD5 may augment the tumor-inducing capabilities of *Agrobacterium*. The chromosomal mis-segregation may contribute to the evolution of fast-growing tumor cells and the DNA breaks induced may act as entry points for integration of the T-DNA.

Materials and Methods

EMSA assay

5' Cy5 labeled primers CEN3FW and CEN3REV, CEN16FW and CEN16REV (**Table 1**) were used to amplify yeast (BY4743) centromeric DNA from chromosomes 3 (CEN3) and 16 (CEN16), respectively. Primers with the same sequences but lacking the 5' Cy5 label were used to obtain the same unlabeled fragments from CEN3 and CEN16 to be used as specific competitor fragments. PCR products were purified (Zymo research D4002) and sent for sequencing. His-tagged VirD5 protein was expressed in *E.coli* strain Rosette2PLySs and purified with Ni-NTA Agarose (QIAGEN: Cat 30230). The EMSA assay was carried out according to Hellman and Fried (2007) with some modifications. The EMSA reaction contained 4µL 5x EMSA buffer (50 mM HEPES pH 7.9, 375 mM KCl, 12.5 mM MgCl2, 0.5 mM EDTA, 5 mM DTT, and 15% Ficoll), 1 µL 1 mg/ml PolydI/dC (Sigma cat: 81349), 1µL 10 mg/ml BSA, 7 µL purified His-tagged VirD5 protein, 6 ng Cy5 labeled probe, and water to a final volume of 20 µL. The mixture was incubated for 30 minutes at room temperature. The reaction was stopped by loading to a 2% agarose gel for electrophoresis in 0.5xTBE buffer at the voltage of 50 V for 7 hours in the cold room, followed by scanning with Perkin Elmer ProExpress 2D Proteomic Imaging System (American Laboratory Trading ALT).

BIFC assay

The pUG34VCn-VirD5 plasmid (or pUG34VCn-VirD5NT) was transformed either with pUG35VNc-Spt4 or pUG35VNc-Dam1 or pUG35VNc-Ipl1 into wild type yeast cells. Transformants were grown at 30 oC on solid MY medium containing methionine to inhibit the expression of VirD5. After 3 days, colonies were transferred to MY liquid medium containing methionine. Overnight cultures washed twice with sterilized water were transferred into new flasks containing MY medium lacking methionine to induce the expression of VirD5. After induction for 1 hour, cells were harvested for BIFC signal visualization using a 63xoil objective on the Zeiss Imager confocal microscope. Images were processed with ImageJ (ImageJ National Institutes of Health).). Plasmids and yeast strains used in this study are listed in **Table 3** and **4**, respectively.

In vitro kinase assay

His-tagged Ipl1 and His-tagged VirD5 were expressed and purified with Ni-NTA Agarose (QIAGEN: Cat 30230). GST-tagged Dam1 protein was expressed in *E.coli* strain Rosette2PLySs. 50 ml cell cultures were centrifuged and resuspended in 1 ml lysis buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA, 1% Triton X-100). After

sonication and centrifugation, the supernatants were stored at -80 °C. The Kinase assay was performed (Keating *et al.*, 2009) by incubating GST-Dam1 (10 µL supernatant from 1ml lysate)-bound glutathione beads, 1 µL purified His-Ipl1 and 8 µL purified His-VirD5 in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% (v/v) β-mercaptoethanol, 0.1 mM EGTA, 10 mM $MgCl_2$, 100 µM ATP and 1 μCi of [γ-^{32}P] ATP for 1 hour at 30 °C. The reaction was stopped by adding 5 µL 4 x sample buffer and boiling for 10 minutes and the mixture was loaded to a 10% SDS-PAGE gel and processed for autoradiography or CBB staining to confirm the equivalent loading of GST-Dam1 protein.

Pull-down assay

GST, GST-Dam1 and GST-Ipl1 were expressed in *E.coli* strain Rosette2PLySs. Equal amounts of the GST-tagged proteins were immobilized on Glutathione HiCap Matrix (Qiagen, 30900) for 2 hours at room temperature, followed by a 3 times washing step with washing buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA). The beads were incubated with purified His-tagged VirD5 protein in binding buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 0.1% Triton X-100) for 2 hours at room temperature. After 3 times washing with buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40), samples were mixed with 20 µL 4x sample buffer and boiled for 10 minutes, followed by centrifugation for 2 minutes at 2000 rpm. Supernatants were loaded to a 10% SDS-PAGE gel for electrophoresis. The presence of the His-tagged VirD5 protein was detected with Anti-His HRP antibodies (Santa Cruz Biotechnology, sc-8036 HRP) by Western Blot analysis.

Chromosome segregation assay

Y716 strain (a gift from Dr Dean Dawson) contains a GFP-LacI and a 256 repeat LacO array integrated in chromosome I. Enrichment of GFP-LacI to the LacO repeats allows the visualization of chromosome I as GFP dot. Wild type and cells integrated with *virD5* or *virD5NT-3NLS* driven by the GAL1 promoter at the URA3 locus were cultured in rich medium containing glucose. Overnight cultured cells were diluted to an OD_{620} of 0.1 and recultured in rich medium containing 2% raffinose and 2% galactose for additional 6 hours. GFP dot signal was observed via a 63xOil objective on the Zeiss Imager confocal. 100 anaphase cells were analyzed for each experiment.

Separation of chromosomes on CHEF gels

Intact yeast chromosomes were isolated in agarose plugs as described from the CHEF kit (Bio-Rad, 170-3591). $6x10^8$ cells overnight cultured yeast cells were washed twice with 0.1 M EDTA (pH 7.5) and resuspended in 630 µL suspension buffer. The suspension mixed with 370 µL 2% low-melt agarose was used to make plugs (10 plugs) for CHEF. Plugs were placed in a 1% agarose gel and sealed with liquid agarose. Electrophoresis was carried out in 0.5xTBE at 14 °C for 24 hours with an initial switch time of 60 s and a final time of 90 s at 200 V. The separated chromosomes were stained with ethidium bromide.

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Table 1. Primers used in this study.

Name	Sequence (5'-3')
Bub2TF	AACGACGTAAAGGTAAAAGAAACAACAGACTTTTAAACTTGTTAA CTTTTGCCGTACGCTGCAGGTCGAC
Bub2TR	CGTTGTAGAATTAAACGATAAAATATAATATTTCTTCACATAGTTT ACGGTATCGATGAATTCGAGCTCG
CEN3FW	CAATATGGAAAATCCACAGAAAGCTATTC
CEN3REV	CCACCAGTAAACGTTTCATATATCCATTC
CEN16FW	CATGGTAGTGATCACAAATAGATCACA
CEN16REV	CAACTGAATAATATTTCTATTTTCGGA
Dam1mycF	CTGTTGCAAAGAAAACTGAAAAAAAAATAAATACAAGGCCCCCCT TCAGACGTACGCTGCAGGTCGAC
Dam1mycR	GCGATATATTTTGTGAGGAGGATAATTCTTTGGTTGGGTTGGGCGT AGTCAATCGATGAATTCGAGCTCG
Dam1FW	CATGCTAGCCATGAGCGAAGATAAAGCTAAAT
Dam1REV	ACGCGTCGACGTCTGAAGGGGGGCCTTGTA
FlagF2	ACGCGTCGACATGGACTACAAGGACGACGACGATAAG
Ipl1FW	GGACTAGTCATGCAACGCAATAGTTTAGT
Ipl1REV	ACGCGTCGACGTAACCGCTTATTTTCCCAAAAGG
Ipl1HisFW	CCATCGATATGCAACGCAATAGTTTAG
Ipl1HisREV	CCCCCCGGGCTATAACCGCTTATTTTCCC
Ipl1TF	AATGCATCCTTGGATACTAAGAAACAAGCCCTTTTGGGAAAATAA GCGGTTACGTACGCTGCAGGTCGAC
Ipl1TR	GGGAGTGATTAATAGTGCCCTTCAAACGATTCTGTCATACTTTAAT TCTAATCGATGAATTCGAGCTCG
pMVHisVirD5FW	GGACTAGTTCACGCTGGGCGTAACCACCA
pMVHisVirD5REV	ACGCGTCGACATTAAAGCCTTCGAGCGTCCC
R1F	ACGCGTCGACGACGATGTGATCTGGCTC
R1R	CCGCTCGAGCCGGCCCGGTTCGCACC
R2F	ACGCGTCGACGGCCGTCCACACGGGATT
R2R	CCGCTCGAGCTGAGCGTCCTCTTGCCA
R3F	ACGCGTCGACAATGACTCTGCGTGGCTTT
R3R	CCGCTCGAGTGGCTCCGCCTCGGCCCAG
R4F	ACGCGTCGACTGCCATGCTGGTTGGATT
R4R	CCGCTCGAGCTGAGCGTCTCCTTGCCA
R5F	ACGCGTCGACGAAAGTGATGCTGTTACG
R5R	CCGCTCGAGGCGTTCCCTCTTACTCCAG
R6F	ACGCGTCGACGATCCCTCAGTCTGGATTG
R6R	CCGCTCGAGTTGAGCTGGCTGCCGCCAT
Rad52F2	AGAGAAGTTGGAAGACCAAAGATCAATCCCCTGCATGCACGCAAG CCTACTCGTACGCTGCAGGTCGAC
Rad52R2	AGTAATAAATAATGATGCAAATTTTTTATTTGTTTCGGCCAGGAAG CGTTTCAATCGATGAATTCGAGCTCG
Spt4TF2	ACCTGGCCACATTCAGTTTGGCAAAAGCGAACGAGGTACAGTGTA AGAGCGTACGCTGCAGGTCGAC

Spt4TR2	CATGTGATATCAGAACGGAAGGTTTTACTCAACTTGACTGCCATCC CTCGGATCGATGAATTCGAGCTCG
SPT4FW	AAAGCGGCCGCTCCAATTTACGTGAAGTAGAT
SPT4REV	CCCCCCGGGACCTTTTTTTTCTAATGAAAGTC
VirD5#1	CCGCCCGGGGATGACAGGAAAG
VirD5#1-2	CGCCTGCAGGACGGGATCGCTG
VirD5#3-2	CGCCTGCAGCGGCGGAACAAGGAC
VirD5#4-2	CGCCTGCAGTCAGCGTTTAAAC
VirD5#9	CCGCCCGGGGGATAAAAACGAAGCCCC
VirD5#10-2	AAAGCGGCCGCTCAGCGTTTAAACGC
VirD5#21	CCATCGATATGACAGGAAAGTCG
VirD5#21-2	CCCCCCGGGTCAGCGTTTAAAC
VirD5#23	GGACTAGTATGACAGGAAAGTCG
VirD5#23-2	ACGCGTCGACTCAGCGTTTAAAC
VirD5#28	CCGCCCGGGATGACAGGAAAGTCG
VirD5#33	AAAGCGGCCGCAAACAGGAAAGTCGAAAGTTC
VirD5#34	AAAGCGGCCGCAACAGGCTGATGCCTCGTTTG
VirD5#38	GGACTAGTATGACAGGAAAGTCGAAAGTTCAC
VirD5#41	CCGCTCGAGTCAGACGGGATCGCTG
VirD5#52	TGCTCTAGATTAGCGTTTAAACGCTTTGTC
VirD5#63	ACGCGTCGACGGAGATATACCATGGGC
VirD5N349R	CCGCTCGAGGATGAGATCTTCCCAGGTC
VirD5N354F	ACGCGTCGACCAGGCCAGGATTGAAAGTG
VirD5N355F	ACGCGTCGACGCCAGGATTGAAAGTGATG

Table 2. Candidate centromeric-kinetochore proteins tested for interaction with VirD5 in BIFC.

Protein name	Function-localization	Interaction
Spt4	Kinetochore, telomere	Y
Ndc10	Inner kinetochore	N
Cep3	Inner kinetochore	N
Ctf13	Inner kinetochore	N
Cse4	Inner kinetochore	N
Mif2	Inner kinetochore	N
Cbf1	Inner kinetochore	N
Mad2	SAC checkpoint	N
Ipl1	MT-Kinetochore regulatory aurora kinase	Y
Dam1	Microtubule-associated protein	Y
Ndc80	Outer kinetochore	N
Scc1	Cohesin	N
Esp1	Separase	N
Skp1	Inner kinetochore	N

Table 3. Plasmids used in this study.

Name	Description	Sources/ references
pMVHis	High-copy vector with a GAL1 promoter and a *URA3* marker.	(van Hemert, *et al*., 2003)
pMVHis-VirD5	VirD5 (XmaI-NotI) was inserted into pMVHis.	This study
pMVHisVirD5ΔR1	VirD5ΔR1 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR2	VirD5ΔR2 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR3	VirD5ΔR3 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR4	VirD5ΔR4 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR5	VirD5ΔR5 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5ΔR6	VirD5ΔR6 (XmaI-XbaI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-202)	VirD5 (1-202) (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505)	VirD5 (1-505) (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR1	VirD5 (1-505) ΔR1 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR2	VirD5 (1-505) ΔR2 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR3	VirD5 (1-505) ΔR3 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR4	VirD5 (1-505) ΔR4 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR5	VirD5 (1-505) ΔR5 (XmaI-XhoI) was inserted into pMVHis.	This study
pMVHis-VirD5 (1-505) ΔR6	VirD5 (1-505) ΔR6 (XmaI-XhoI) was inserted into pMVHis.	This study
pRS315	Single-copy yeast plasmid with a *LEU2* marker.	(Sikorski and Hieter, 1989)
pRS315-Spt4	Spt4 including its own promoter and terminator (NotI-XmaI) was inserted into pRS315.	This study
pUG34GFP	Single-copy plasmid with a *HIS3* maker for a fusion with the C-terminus of GFP driven by the MET25 promoter.	(Sakalis, 2013)
pUG34-GFP-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34GFP.	This study
pUG34-GFP-VirD5NT (1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34GFP.	This study
pUG34-2xGFP-VirD5NT (1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34-2xGFP.	This study
pUG34-GFP-VirD5NT (521-833)	VirD5 (521-833) (SpeI-SalI) was inserted into pUG34GFP.	This study
pET16H	pBR322 base plasmid with an N-terminal10xHis tag under the control of the T7 promoter.	Novagen
pET16H-VirD5	VirD5 (ClaI-XmaI) was inserted into pET16H.	This study
pET16H-Ipl1	Ipl1 (ClaI-XmaI) was inserted into pET16H.	This study
pGEX-KG	pMB1 based plasmid with an N-terminal GST tag under the control of the TAC promoter.	(Guan and Dixon, 1991)
pGEX-KG-Ipl1	Ipl1 (SpeI-SalI) was inserted into (XbaI-SalI) of pGEX-KG.	This study
pGEX-KG-Dam1	Dam1 (NheI-SalI) was inserted into(XbaI-SalI) of pGEX-KG.	This study

pUG34VCn	Single-copy plasmid with an N-terminal fusion with the C-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG34VCn-VirD5	VirD5 (SpeI-SalI) was inserted into pUG34VCn.	This study
pUG34VCn-VirD5(1-505)	VirD5 (1-505) (SpeI-XhoI) was inserted into pUG34VCn.	This study
pUG34VCn-VirD5(1-505) ΔR5	VirD5 (1-505) ΔR5 (SpeI-XhoI) was inserted into pUG34VCn.	This study
pUG35VNc	Single-copy plasmid with an C-terminal fusion with the N-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
pUG35VNc-Ipl1	Ipl1 (SpeI-SalI) was inserted into pUG35VNc.	This study
pUG35VNc-Dam1	Dam1 (NheI-SalI) was inserted into (SpeI-SalI) of pUG35VNc.	This study
pRS425	High-copy plasmid with a *LEU2* marker.	(Christianson *et al*., 1992)
pRS425-pGAL1-VirD5(1-505)	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5(1-505) as template was inserted into SpeI-SalI of pRS425.	This study
pRS425-Glc7	Glc7 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Glc8	Glc8 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Gip3	Gip3 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS425-Gip4	Gip4 including its own promoter and terminator was inserted into XmaI- NotI of pRS425.	This study
pRS305-pGal1-VirD5	pGal1-His-VirD5-Ter PCR using pMVHis-VirD5 as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pGal1-VirD5NT-3xNLS	pGAL1-His-VirD5 (1-505)3xNLS-Ter PCR using pMVHis-VirD5NT as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pMET25-GFP	PCR of pMET25-GFP using pUG34GFP as template was inserted into SpeI-SalI of pRS305.	This study
pRS305-pMET25-GFP-VirD5NT	PCR of pMET25-GFP PCR using pUG34GFP as template was inserted into SpeI-SalI of pRS305.	This study
pRS306-pGAL1-VirD5	pGAL1-His-VirD5-Ter PCR using pMVHis-VirD5 as template was inserted into SpeI-SalI of pRS306.	This study
pRS306-pGAL1-VirD5NT-3xNLS	pGAL1-His-VirD5 (1-505)3xNLS-Ter PCR using pMVHis-VirD5NT as template was inserted into SpeI-SalI of pRS306.	This study

Table 4. Yeast strains used in this study.

Yeast strain	Genotype	Sources/ references
BY4743	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0)	(Brachmann *et al*., 1998)
BY4743-Δspt4	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0- Δspt4-KanMX- Δspt4-KanMX)	Eurosarf
BY4743-HTA2-CFP	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX)	(Sakalis, 2013)
BY4743-HTA2-CFP-VirD5	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX, pGAL1-His-VirD5-LEU2, pGAL1-His-VirD5-LEU2)	This study
BY4743-HTA2-CFP-GFP	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,pMET25-GFP-LEU2, pMET25-GFP-LEU2)	This study
BY4743-HTA2-CFP-GFP-VirD5NT	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,pMET25-GFP-VirD5 (1-505)-LEU2, pMET25-GFP-VirD5 (1-505)-LEU2)	This study
BY4743-HTA2-CFP (pMVHis)	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,(pMVHis-URA3))	This study
BY4743-HTA2-CFP (pMVHis-VirD5NT)	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0,HTA2-CFP-KanMX, HTA2-CFP-KanMX,(pMVHis-VirD5 (1-505)-URA3))	This study
BY4743-VirD5	(MATa-α his3Δ1-his3Δ1 leu2Δ0-leu2Δ0 LYS2-lys2Δ0 met15Δ0-MET15 ura3Δ0-ura3Δ0, pGAL1-His-VirD5-LEU2)	This study
BY4741	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0)	(Brachmann *et al*., 1998)
BY4741-Dam1-3xGFP	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0,Dam1-3xGFP-KanMX)	This study
BY4741-Dam1-3xGFP-VirD5	(MATa his3Δ1- leu2Δ0 -met15Δ0- ura3Δ0,Dam1-3xGFP-KanMX-pGAL1-His-VirD5-LEU2)	This study
Y716	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2[lacO256 LEU2])	(Meyer *et al*., 2013)
Y716-VirD5	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2[lacO256 LEU2]; pGAL1-His-VirD5-URA3)	This study
Y716-VirD5NT-3xNLS	(leu2-?, lys2-pLL1[PCYC1-GFP-lacI LYS2], met13-c, tyr1-2, MATa, ura3-1, trp1-Δ63, cyh2-1, his3-Δ1, CEN1-pJN2 [lacO256 LEU2]; pGAL1-His-VirD5(1-505)3xNLS-URA3)	This study
MAS101	S. cerevisiae pRS306 [PHIS3-GFP-TUB1]	(Straight *et al*., 1997)
MAS101-VirD5	MAS101- pGAL1-His-VirD5-LEU2	This study
W303a	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15)	(Thomas and Rothstein, 1989)
W303a-Rad52-GFP	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP)	This study
W303a-Rad52-GFP-VirD5	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP,pGAL1-His-VirD5-URA3)	This study

W303a-Rad52-GFP-VirD5NT-3xNLS	(MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15, Rad52-GFP, pGAL1-His-VirD5 (1-505)-URA3)	This study
YB3039	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ	(Norden *et al.*, 2006)
YB3039-Δspt4	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ,Δspt4	This study
YB3039(34VCN)	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ (pUG34VCn)	This study
YB3039(34VCN-VirD5)	MATa,IPL1-3GFP-URA3,ura3-52,lys2-801,ade2-101,his3Δ200,trp1Δ (pUG34VCn-VirD5)	This study

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Chapter 4

The VirD5 protein interferes with mitosis in plant cells

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Abstract

Agrobacterium tumefaciens is a plant pathogen that causes crown gall disease in dicotyledonous plants at wound sites, by transferring a fragment of DNA (T-DNA) from its tumor-inducing (Ti) plasmid into the host genome. During infection, several virulence proteins encoded by the Ti plasmid are also transferred independently from the T-DNA from the bacterium to host cells via a Type Four Secretion System (TFSS). The VirD5 protein is one of these translocated virulence proteins, but its function in the transformation process is still elusive up to date. Here, we generated transgenic plants expressing VirD5 under the control of a tamoxifen inducible promoter, and found that expression of the protein inhibited plant growth. Further molecular experiments demonstrated that VirD5 targets the host mitosis regulatory Aurora kinases, causing chromosome mis-segregation and aneuploidy formation during mitosis. Aneuploidy is a hallmark of tumor cells, suggesting a possible role of VirD5 in the process of crown gall tumor formation. A Cre/*lox*-GAL4/UAS double inducible system was employed to express VirD5 specifically in tapetum cells, which are important for the development of pollen and we found that this generated male-sterile plants, a trait of economic value.

Introduction

Agrobacterium tumefaciens, a rod shaped, Gram-negative soil bacterium, is the causal agent of the crown gall disease in plants (Smith and Townsend, 1907), which is characterized by the formation of neoplastic overgrowths on roots, the root crown and stems. Crown gall cells contain a segment from the *Agrobacterium* Ti plasmid, called the T-DNA, which is responsible for the tumorous properties of the crown gall cells. Genetic transformation is initiated in response to phenolic compounds produced by plant cells at wound sites (Huang *et al*., 1990). Then the VirD2 protein of the bacterium is produced, which nicks the Ti plasmid at the 24 bp direct repeats that surround the T-DNA ultimately leading to the generation of single-stranded copies of this transferred DNA (T-DNA) which are called T-strands (Stachel *et al*., 1987). VirD2 remains covalently bound to the 5' end of the T-strand, thus forming a T-complex (Ward and Barnest, 1987), which is delivered into host cells via the VirB Type Four Secretion System (TFSS). The T-strand is converted into a double stranded molecule in the plant cells and is integrated into the host genome by the host DNA recombination machinery predominantly via non-homologous recombination (Tinland *et al*., 1995). Expression of the genes present on the T-DNA in plant cells promotes uncontrolled growth and division leading to tumorigenesis.

Besides the T-complex, several virulence proteins encoded by the Virulence region of the Ti plasmid are transferred into host cells independently of the T-complex via the same VirB TFSS apparatus. These translocated Vir proteins including VirE2, VirE3, VirF and VirD5 contain a positively charged C-terminus which is essential for protein translocation (Vergunst *et al*., 2005). How these translocated effector proteins assist in plant transformation is only partially known. The VirE2 protein, a single-strand DNA binding protein can bind along the length of the T-strand (Citovsky *et al*., 1990) and thus protect the T-strand from degradation by host nucleases (Citovsky and Zambryski, 1989). It also interacts with the host proteins VIP1 and VIP2, which together with VirE2 assist in the nuclear uptake of the T-complex and their subsequent targeting to the host chromatin (Tzfira and Citovsky, 2000). The VirE3 protein may function as a transcription factor as it interacts with the plant-specific general transcription factor Brp, a homolog of TFIIB (García-Rodríguez, Schrammeijer, and Hooykaas, 2006). The VirF protein is a host range factor and one of the very few prokaryotic proteins containing an F-box motif, with which it physically can interact with plant SKP1-like proteins and form a SCF-ubiquitination complex. It is thought to play an important role in the decoating of VirE2 from the T-strand before insertion into the host chromosome (Schrammeijer *et al*., 2001; Tzfira, Vaidya, and Citovsky, 2004). In some host plants a host encoded F-box protein, may substitute for VirF.

The VirD5 protein is translocated through the TFSS into plant cells, where it moves into the nucleus of the host cells (Vergunst *et al*., 2005), but the function of this protein is still elusive. It was published that the VirF protein may be stabilized in host cells via physical interaction with VirD5 thus promoting T-complex decoating (Magori and Citovsky, 2011). In another recent report it was however suggested that VirD5 competes with VBF for interacting with VIP1 thus preventing the decoating of the T-complex. Besides, it was suggested that VirD5 might play an important role as a transcription factor in the transactivation of host genes (Wang *et al*., 2014).

In our previous studies it was shown that expression of VirD5 in yeast cells is toxic and leads to growth arrest. Further molecular analysis showed that the protein was localized at the centromeres/kinetochores of the yeast chromosomes in the nucleus through an interaction with the Spt4 protein, which is conserved throughout eukaryotes. The VirD5 protein interacted with the yeast Ipl1/Aurora kinase at the kinetochores and was able to stimulate the activity of this kinase *in vitro*. The yeast Ipl1/Aurora kinase plays a central role at the kinetochores in the control of proper kinetochore-microtubule attachment by phosphorylating proteins at this interaction site to mediate detachment as long as not all kinetochores have correct attachments. The presence of VirD5 eventually led to cell arrest and upon recovery aneuploid cells were recovered indicative of chromosome mis-segregation (**Chapter 2** and **3**).

The Ipl1/Aurora kinase is a conserved serine/threonine protein kinase that is present in plant and mammalian cells as well (Buvelot *et al*., 2003; Zimniak *et al*., 2012). Besides for the correction of erroneous chromosome-microtubule attachments it has several other functions during mitosis. In plants and mammalian cells the functions of Ipl1 have been divided over three related Aurora kinases. In mammalian cells these are called Aurora A, Aurora B and Aurora C (Fu *et al*., 2007) and in plant cells, Aurora1, Aurora2 and Aurora3 (Van Damme *et al*., 2011). The Aurora kinases from *Arabidopsis thaliana* exhibit different sublocations in the cell, indicating their different functions in mitosis (Kawabe *et al*., 2005). Ectopic expression of these Aurora kinases disrupts proper chromosome segregation in both meiosis and mitosis (Demidov *et al*., 2014). Here, we surveyed how the *A. tumefaciens* virulence protein VirD5 affected cell division in *A. thaliana* and whether it interacted with the three plant Aurora kinases. We found that also in plants expression of VirD5 leads to chromosome mis-segregation during mitosis.

Results

Generation of transgenic plants expressing VirD5

As VirD5 is a translocated virulence protein, we studied its effects in plant cells using transgenic plants with an inducible expressing construct. To this end, a T-DNA containing *virD5* under the control of a tamoxifen inducible promoter was inserted in the *A. thaliana* genome by floral dip transformation with *Agrobacterium*. T1 primary transformants were selected on MS medium with kanamycin. These exhibited slight growth retardation even at non-induction conditions as compared with plants transformed with the empty T-DNA, indicating that the promoter was somewhat leaky and that VirD5 was toxic for plants (data not shown). The T2 offspring from 22 independent T1 plants were germinated on MS medium containing kanamycin together with or without 10 μM tamoxifen. None of the seedlings could grow in medium with both kanamycin and tamoxifen, while approximately 75% of the seedlings kept growing in medium containing kanamycin alone (**Figure 1A**), suggesting that expression of VirD5 is lethal to plant cells, which is in line with previous work showing that VirD5 inhibits yeast cell growth (**Chapter 2**). One of these transgenic lines containing a single T-DNA insertion was characterized in some more detail and used in the subsequent experiments (**Figure 1B**).

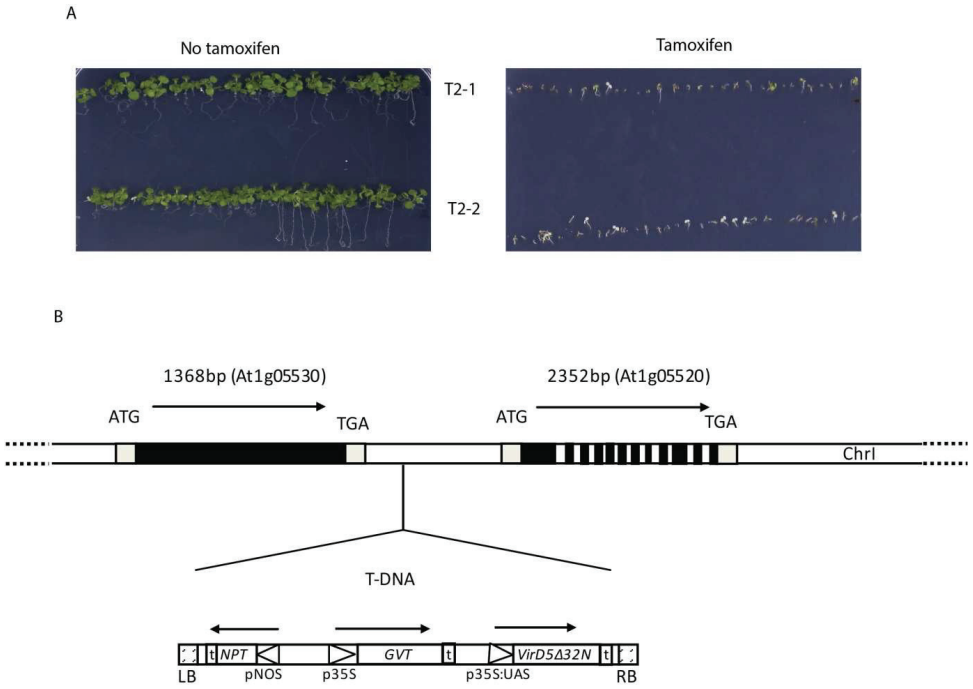


Figure 1. Growth inhibition of transgenic *A. thaliana* expressing the *A. tumefaciens* virulence protein VirD5. (A) T2 seedlings from two independent transgenic lines containing the *virD5* gene under the control of a tamoxifen inducible promoter were germinated on kanamycin containing MS medium with or without 10 μM tamoxifen. (B) The T-DNA insertion site in one of the 22 independent transgenic lines was determined by sequencing after TAIL-PCR. GVT, a fusion protein containing GAL4-VP16-estrogen receptor; LB, T-DNA left border; pNOS, NOS promoter; p35S, CaMV35S promoter; RB, T-DNA right border; t, terminator; VirD5Δ32N, VirD5 protein lacking the N-terminal 32 amino acids. Arrows indicate the orientation of transcription.

VirD5 targets mitosis via the Aurora kinases

We previously studied the function of VirD5 in yeast cells and found that this protein is located in the nucleus at the centromeres/kinetochores of the yeast chromosomes and there interacts with the yeast Aurora kinase Ipl1. The Aurora kinase family is a well-conserved serine/threonine protein kinase family that plays an essential role in the control of appropriate kinetochore-microtubule attachments during mitosis (Andrews *et al*., 2003; Buvelot *et al*., 2003; Demidov *et al*., 2014). In plants, there are three Aurora kinases, namely Aurora1, Aurora2 and Aurora3. To test whether VirD5 can interact with these kinases, we carried out an *in vivo* Bimolecular Fluorescence Complementation (BIFC) assay in *A. thaliana* protoplasts. A robust YFP fluorescence was seen in the nuclei of cells in which both VirD5 and one of the Aurora kinases was present indicating that all three plant Aurora kinases had strong interactions with VirD5 and exclusively in nucleus (**Figure 2**). In contrast neither the presence of VirD5 nor Aurora kinases alone did lead to a reconstitution of the fluorescent signal. In order to find out whether these interactions were direct, we performed an *in vitro*

pull-down experiment: His-tagged VirD5 was incubated with either empty GST or GST-tagged Aurora1/Aurora2/Aurora3 for 2 hours in binding buffer at room temperature, and after 3 times washing, proteins were separated in SDS-PAGE for western blot analysis. As can be seen in **Figure 3**, VirD5 bound to all three plant Aurora kinases *in vitro*, but not to the empty GST beads, indicating that VirD5 binds directly to the Aurora kinases.

VirD5 interacts with the plant Spt4 protein

We found that VirD5 physically interacts with yeast Spt4 (**Chapter 2**). In *A. thaliana*, there are two genes encoding Spt4, *SPT4-1* (At5g08565) and *SPT4-2* (At5g63670), which share high homology with that of the yeast and mammalian Spt4. In order to determine whether these two Spt4 proteins interact with VirD5, we carried out a BIFC assay in yeast cells. Only the plant Spt4-1 protein strongly interacted with VirD5 (**Figure 4A**). As previous results showed that the deletion of *spt4* in yeast suppressed the lethality of VirD5 (**Chapter 2**), we

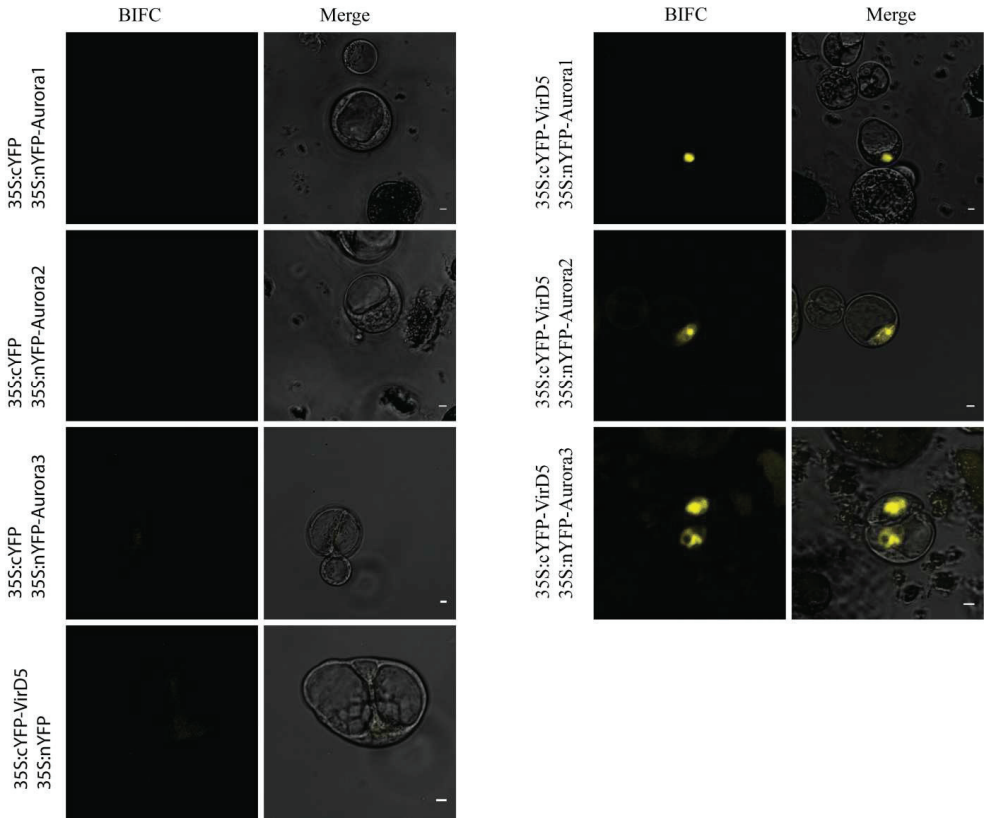


Figure 2. A Bimolecular Fluorescence Complementation (BIFC) assay for interactions between VirD5 and Aurora kinases in *Arabidopsis thaliana* protoplasts. 35S, Cauliflower mosaic virus promoter. nYFP, N-terminus of YFP (1-154aa). cYFP, C-terminus of YFP (155-813aa). Scale bar, 5μm.

wondered whether the plant Spt4-1 protein can restore the lethality of VirD5 in the yeast *spt4* deletion mutant. To this end we cotransformed a plasmid encoding VirD5 driven by the GAL1 promoter either with empty plasmid or plasmid encoding Spt4-1 under the control of the MET25 promoter in the yeast *spt4* deletion mutant. In the presence of *A. thaliana* Spt4-1 VirD5 was somewhat more toxic in the yeast *spt4* mutant background (**Figure 4B**), suggesting that the *A. thaliana* Spt4-1 protein may partially have a similar biological role in plant cells as Spt4 in yeast.

Presence of VirD5 reduces cell division in the Arabidopsis root meristem

Since Aurora kinases play vital roles in mitosis via phosphorylation of substrates involved in cell division, (Yamagishi *et al.*, 2010), this raises the possibility that VirD5 might affect plant cell division. In order to test this we grew *A. thaliana* seedlings homozygous (**Figure 5**) for a construct containing the *virD5* gene driven by a tamoxifen inducible promoter on MS media to which different concentrations of tamoxifen had been added. When tamoxifen was present at concentrations higher than 1 μM, growth of the seedlings stopped immediately (data not shown). However, on media with lower doses (0.2 μM and 0.5 μM) of tamoxifen growth continued slowly. Such seedlings had a shorter root meristematic region when compared to the same homozygous plants treated with DMSO instead of tamoxifen as a control (**Figure 6**). The short root meristem could reflect problematic mitosis, due to erroneous chromosome segregation.

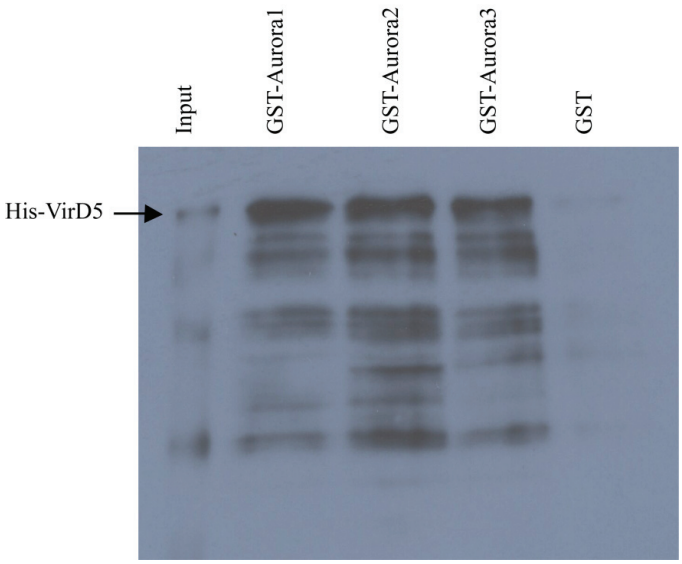


Figure 3. GST pull-down assay for interactions between VirD5 and Arabidopsis Aurora kinases. His-tagged VirD5 was incubated with either empty GST or GST-tagged Aurora1/2/3 *in vitro*. The presence of His-VirD5 was detected by anti-His antibody.

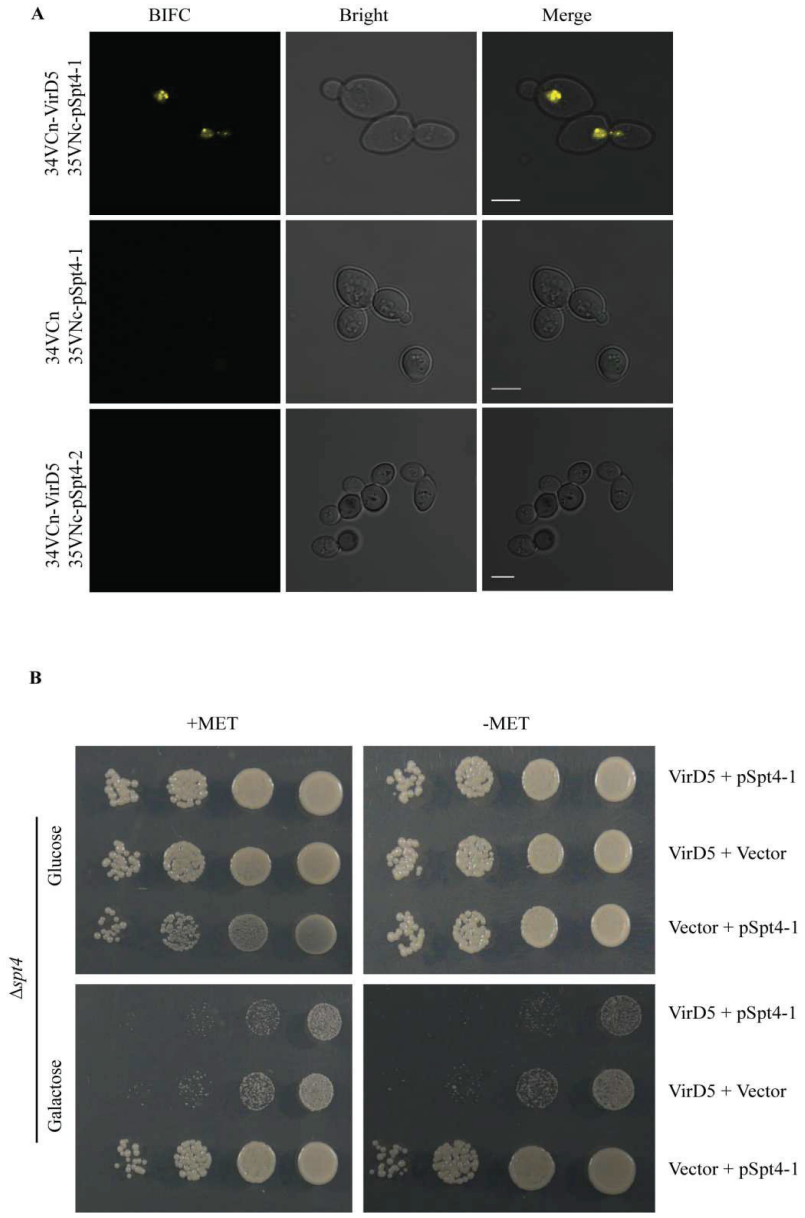


Figure 4. VirD5 interacts with plant Spt4-1. (A) Yeast cells transformed with BIFC vectors. 34VCn, the C-terminus of YFP fragment (VC173) fused with the N-terminus of testing proteins. 35VNc-pSpt4-1 and 35VNc-pSpt4-2, the N-terminus of YFP fragment (VN173) fused with the C-terminus of Spt4-1 and Spt4-2 proteins from *A. thaliana*. Scale bar, 5 μm. (B) Plasmid containing *virD5* under the control of the GAL1 promoter was cotransformed with either empty vector or vector encoding plant *SPT4-1* driven by the MET25 promoter which is active in methionine free medium in the yeast *spt4* deletion mutant. Transformants were serially diluted and spotted onto minimal medium containing glucose or galactose with or without methionine.

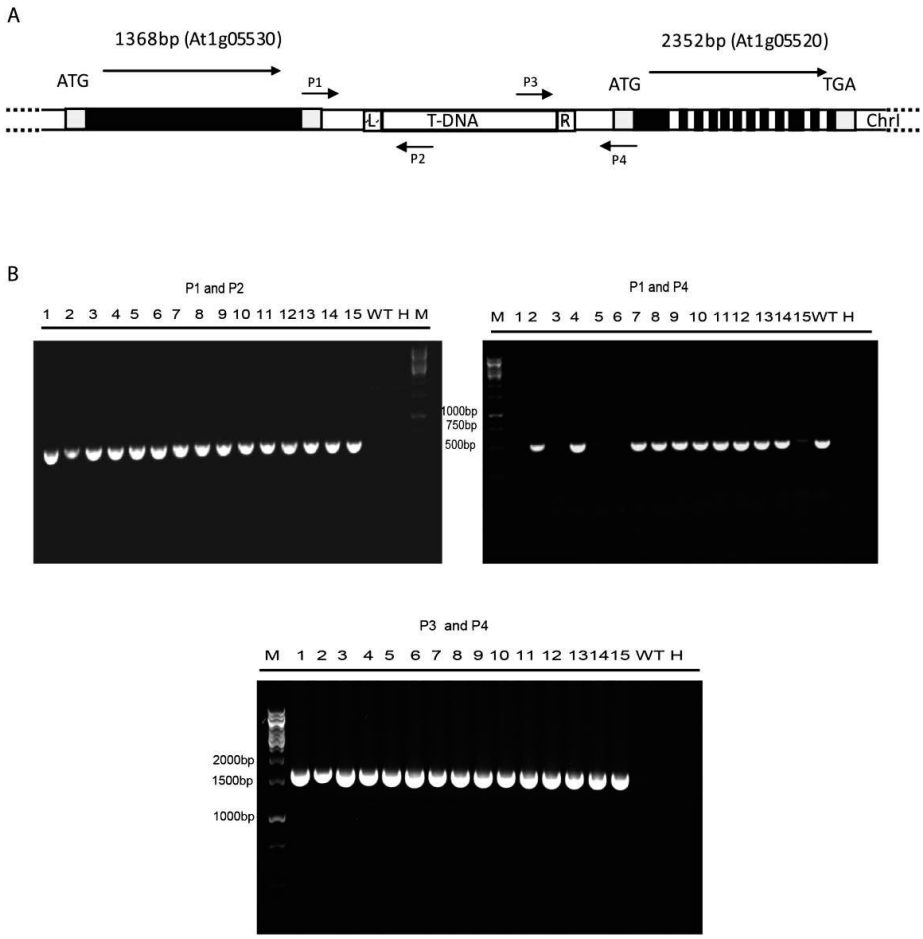


Figure 5. Genotype analysis of the offspring of a plant containing the construct for VirD5 driven by the tamoxifen inducible promoter. (A) Scheme of the T-DNA insertion and primers used to determine the presence or absence of the T-DNA in each of the two copies of chromosome 1. (B) The fifteen individual T3 plants, progeny from one of the hemizygous T2 plants, and WT plants were analyzed by PCR for being hemizygous or homozygous for the T-DNA. Plant line 6, one of the plants homozygous for the T-DNA, was used for further experiments.

In order to follow chromosome segregation directly in plant cells we used a plant line containing an array of 256 copies of the *lac* operator (LacO) in its genome on chromosome 5 that could be visualized under the microscope as a bright green fluorescent dot by expression of GFP-LacI. The cells also expressed a H2B-DsRed fusion protein, to mark the nucleus with red fluorescence (Matzke *et al*., 2010). This chromosome marked homozygous plant line was crossed with a homozygous transgenic *A. thaliana* line containing the *virD5* gene driven by the tamoxifen inducible promoter. F1 seeds of this cross were geminated on MS medium without tamoxifen first. Subsequently, 4 days after germination seedlings were moved to liquid MS medium containing 10 μM tamoxifen and incubated for an additional 24 hours. Root cells from the meristematic zone were chosen for analysis because of their lower

background fluorescence. As can be seen in **Figure 7**, root cells from F1 heterozygous plants from a cross of the LacO/GFP-LacI line with the wild type showed mainly nuclei with a single bright GFP dot, and in a few mitotic cells before cytokinesis two dots. In contrast in root cells from F1 plants from the cross with the VirD5 expressing line, besides many cells with a single bright dot cells displaying more than two bright dots were visible, illustrating chromosome mis-segregation and generation of aneuploid cells. Besides, we also used another chromosome marked plant line to avoid a possible chromosome bias, but a similar pattern of multiple fluorescent dots were visible also in this line when VirD5 was expressed (data not shown), suggesting that VirD5 causes chromosome mis-segregation in plant cells during cell division.

Expression of VirD5 from the T-DNA inhibits plant tumor formation

Agrobacterium tumefaciens causes crown gall disease by transferring an oncogenic segment from its Ti plasmid into plant cells at wound sites. This T-DNA is inserted randomly into the host genome; expression of genes present on the T-DNA in the transformed plant cells leads to overproduction of plant growth regulators and cell division, ultimately resulting in the formation of a crown gall tumor. Translocated virulence effector proteins contribute to tumor initiation by enhancing the efficiency of T-DNA delivery and by inhibiting host defense responses. The translocated VirD5 protein causes chromosome mis-segregation in eukaryotic cells and thus inhibits cell division as shown above. Chromosomal abnormalities and aneuploidy are common in human tumors and have also been described for crown gall tumor cells. Therefore, we tested how VirD5 affected tumor formation.

First we compared tumor formation by the *virD5* mutant in comparison with the wild type. To this end we inoculated *Nicotiana glauca* with wild type *Agrobacterium* strain LBA1010 and *virD5* deletion mutant strain LBA3550. As shown in **Figure 8**, the *Agrobacterium* strain lacking VirD5 induced tumors of equal size as the wild type strain expressing and translocating VirD5. To test whether overexpression of VirD5 had an influence on tumor formation, we cloned the wild type *virD5* gene under the control of the 35S promoter in the

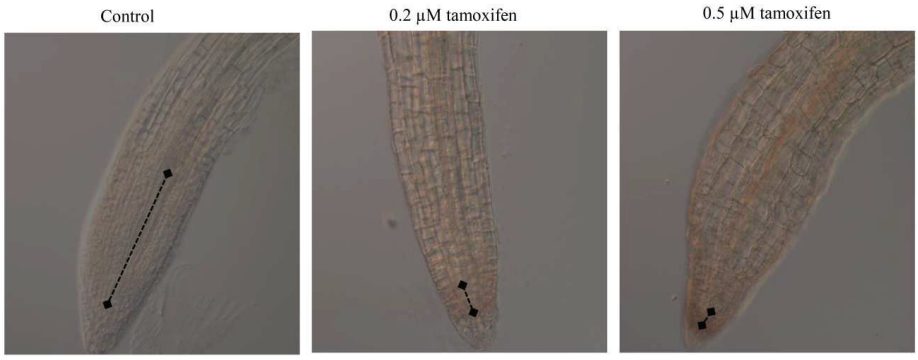


Figure 6. VirD5 disrupts cell division in the root meristem. Homozygous transgenic *A. thaliana* plants containing *virD5* driven by the tamoxifen inducible promoter were germinated on MS medium with addition of tamoxifen (middle and right) or DMSO (left). Dotted line reveals the size of the meristematic zone.

T-region of a binary vector, and transformed this into both LBA1010 (wild type) and LBA3550 (*virD5* mutant), resulting in LBA1010 (35S:VirD5) and LBA3550 (35S:VirD5), respectively. These two strains were inoculated onto *N. glauca,* and after three weeks tumor formation was scored. We observed that only extremely small tumors were induced by both strains expressing VirD5 in the T-DNA (**Figure 8**). This was in line with the negative effects on cell division observed in the transgenic plants expressing VirD5 from an inducible promoter. It also indicates that the dosage of VirD5 needs to be carefully controlled for this conserved protein to be able to contribute to tumor formation.

Tumor formation on A.thaliana roots is influenced by the dose of VirD5

As an alternative for the tumor assay on plants stems we performed a more quantitative tumor assay on *A. thaliana* root segments. To this end, we inoculated *A. thaliana* roots with wild type *Agrobacterium* strain LBA1010, *virD5* deletion mutant strain LBA3550 and wild type strain with a plasmid encoding VirD5 under the control of the *tac* promoter, LBA1010 (VirD5). As can be seen in **Figure 9**, the *virD5* deletion strain induced slightly less calli than the wild type strain expressing VirD5. However, the wild type strain with the additional expression of VirD5 present on the plasmid induced much less calli. This indicates that VirD5 contributes to tumor formation, but that the dosage of VirD5 needs to be well balanced for optimal tumor development.

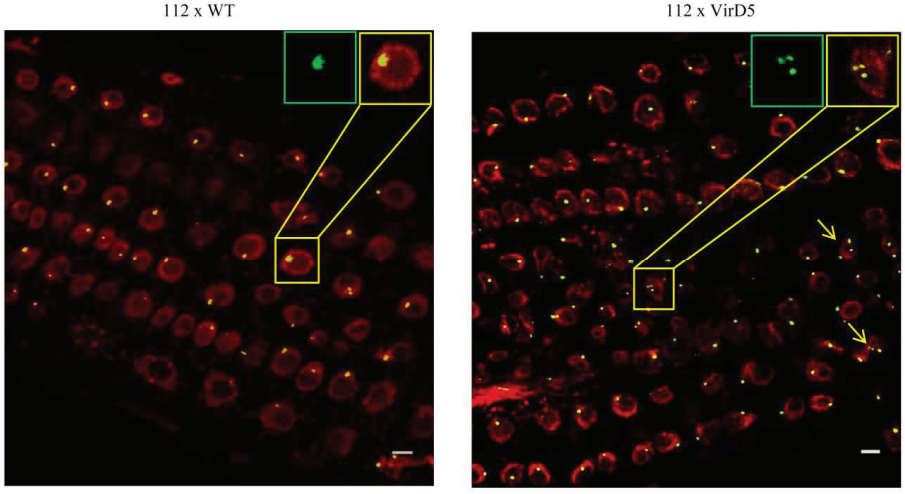


Figure 7. Chromosome mis-segregation due to expression of VirD5 in root tip cells. In the cells one copy of chromosome 5 is marked with 256 repeats of the *lac* operator and visualized by expression of GFP-LacI, while the nuclei are visualized by expression of H2B-dsRed. Left: single dots present in the cells in the absence of VirD5 are indicative of normal chromosome segregation. Right: multiple dots (yellow arrows) in cells in the presence of VirD5 reveal chromosome mis-segregation. 112 represents chromosome 5 marked homozygous plant line containing 256 repeats of the *lac* operator, *gfp-lacI* and *H2B-dsRed* genes under the control of 35S promoter. WT represents wild type plant line. VirD5 represents homozygous transgenic plant line inserted with *virD5* driven by the tamoxifen inducible promoter. 50 plants from each group were counted. Scale bar, 5μm.

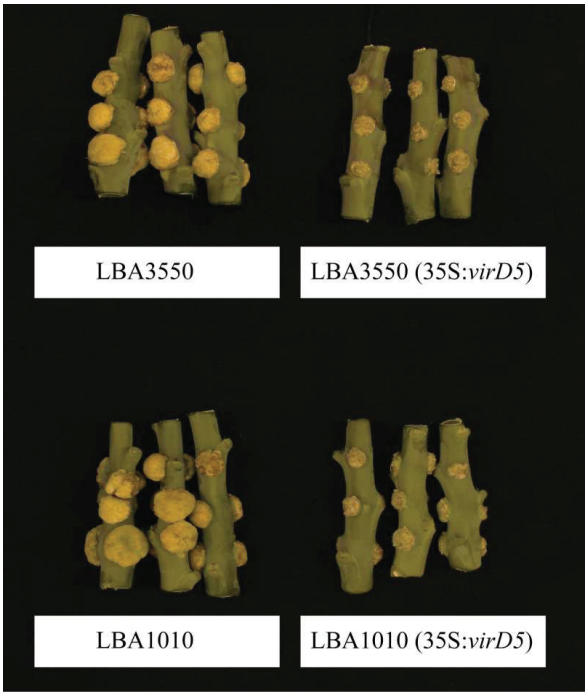
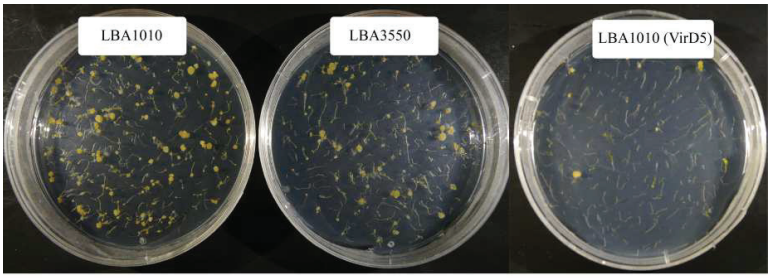


Figure 8. Tumor assay in *Nicotiana glauca*. LBA1010, a wild type *Agrobacterium* strain. LBA3550, LBA1010Δ*virD5* strain. 35S:VirD5, a binary vector containing the *virD5* gene under the control of the 35S promoter in its T-DNA region. Tumors were photographed after 3 weeks.

Application of the toxic property of VirD5 for cell ablation

Male sterile plants are widely utilized by plant breeders, and these have also been obtained by genetic engineering by expressing a cell-autonomous toxic gene in the tapetum, the cell layer feeding the male gametophytic cells (Mariani *et al*., 1990). As VirD5 inhibited cell division by disturbing chromosome segregation, we expected that VirD5 could be used as a cell-autonomous toxic gene for similar purposes. In these experiments, we made use of the CRE/*lox* system in combination with the GAL4/UAS system to tightly control the expression of VirD5 exclusively in the tapetum cells (**Figure 10**). To this end, we constructed a binary plasmid containing the tapetum specific promoter A9 of *A. thaliana* (Paul *et al*., 1992) driving the expression of CRE specifically in tapetum cells. The *virD5* gene was cloned behind a UAS cassette in the T-region of a second binary vector that also contained a construct consisting of the open reading frame of the transcriptional activator Gal4VP16 driven by the 35S promoter but which was interrupted by the CRT1 selection marker (Norihiko *et al*., 1993) flanked by two *lox* recombination sites thus preventing expression of GAL4VP16. Both binary plasmids were separately transformed into *Agrobacterium* strains and ultimately transformed by these into the *A. thaliana* genome via flora dip. A single copy line of each transformation was used for a cross that would bring the two T-DNAs together in the cells of the offspring.



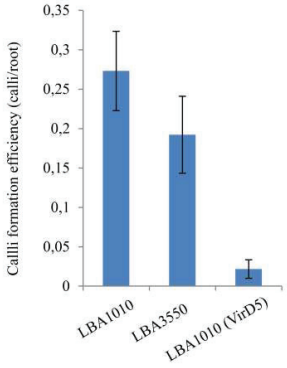


Figure 9. Tumor formation on *A. thaliana* roots. Roots of *A. thaliana* plants were infected with wild type *Agrobacterium* strain LBA1010, *virD5* deletion strain LBA3550, and wild type strain with a plasmid encoding *virD5* under the control of a *tac* promoter LBA1010 (VirD5). Photos were taken 3 weeks after infection. Error bars represent the mean ± SE from three independent experiments.

In order to prevent the expression of CRE and thus of VirD5 during crossing, we used pollen from plants containing *virD5* driven by UAS to fertilize flowers of plants harboring the *cre* gene under the control of the A9 promoter. F1 seedlings containing both constructs were selected on MS medium containing 15 mg/L hygromycin and 1 μM norflurazon, a herbicide to which the *Erwinia* CRT1 gene provides resistance. In total, 24 positive F1 seedlings were selected in this way for further experimentation. These developed into normal mature plants, but 10 out of 24 of these plants formed no or only smaller siliques, as can be seen in **Figure 11A**, whereas control lines, which were derived from wild type plants crossed with UAS-VirD5 plants, formed siliques of a normal size. This suggested that many of the plants containing both of the T-DNAs formed reduced amounts of seeds. To further understand this phenotype, we surveyed open flowers of plants expressing VirD5 in the tapetum cells and found that the stamina of these flowers were significantly reduced in length (**Figure 11B**) and that there were less or no pollen present in the anthers, in comparison with flowers from normal control plants. In order to test the viability of pollen from both control and VirD5 active plants, we performed pollen Alexander staining; this revealed that VirD5 expressing plants produced much less viable pollen grains than wild type

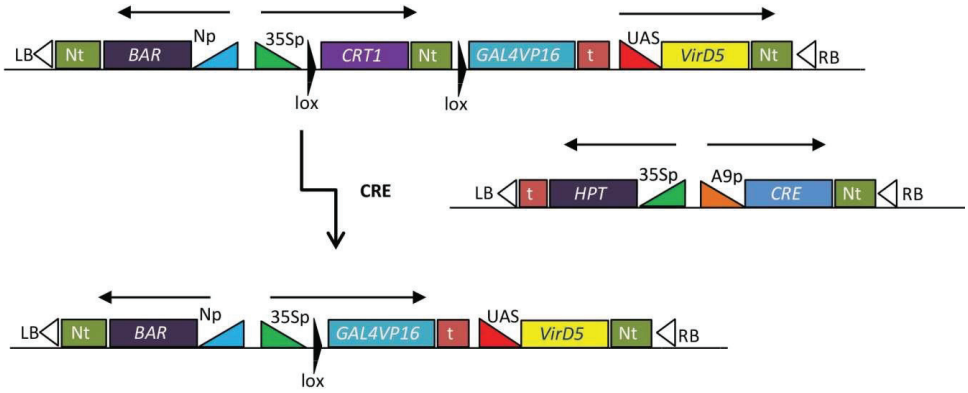


Figure 10. Schematic representation of Cre-mediated excision of the sequences between the directly repeated *lox* recombination sites, leading to the expression of VirD5 specifically in tapetum cells. Arrows indicate the directions of transcription. A9p, promoter from the *A. thaliana* tapetum-specific *A9* gene; BAR, herbicide bialaphos resistance gene; CRE, Cre recombinase; CRT1, phytoene desaturase from *E.uredovora* for selection of norflurazon-resistant plants; HPT, hygromycin phosphotransferase gene; LB,T-DNA left border; Np, NOS promoter; Nt, NOS terminator; RB, T-DNA right border; t, terminator from the 35S transcript of CaMV.

plants. As shown in **Figure 11C**, only half of the pollen grains in the anthers colored red, indicating that they were viable. These results illustrated that expression of VirD5 in the tapetum cells interfered with pollen formation, and thus with seed formation.

Discussion

Agrobacterium tumefaciens induces plant tumors by transferring T-DNA as well virulence proteins into plant cells at wound sites. The expression of genes on the T-DNA triggers the uncontrolled growth of plant cells and thereby causes the crown gall disease. How the translocated effector proteins contribute to tumorigenesis is not fully understood. In this study, we demonstrated that one of the translocated virulence proteins called VirD5 affects tumor formation in a dose dependent way.

In our earlier studies in yeast *Saccharomyces cerevisiae*, we could show that VirD5 caused DNA damage and also that it bound to the yeast Aurora B/Ipl1kinase and stimulated its kinase activity, consequently triggering chromosome mis-segregation (**Chapter 3**). In view of the high conservation of the Aurora kinases in different eukaryotes, we tested the potential interaction of VirD5 with the three Aurora kinases from *A. thaliana* via *in vivo* BIFC and *in vitro* pull-down assays, and found that VirD5 indeed could interact with all the plant Aurora kinases (**Figure 2** and **3**).

Given this observation, we next examined a possible effect on plant cell division of VirD5. Homozygous plants containing *virD5* controlled by the tamoxifen-inducible promoter died in the presence of tamoxifen or at lower concentration of the inducer showed a growth inhibition (**Figure 1** and **6**). Previously, we found that the expression of VirD5 in yeast affected cell division and in the end stopped cell growth (**Chapter 2**). Therefore, we examined the development of root tips of *A. thaliana*, containing many mitotically active

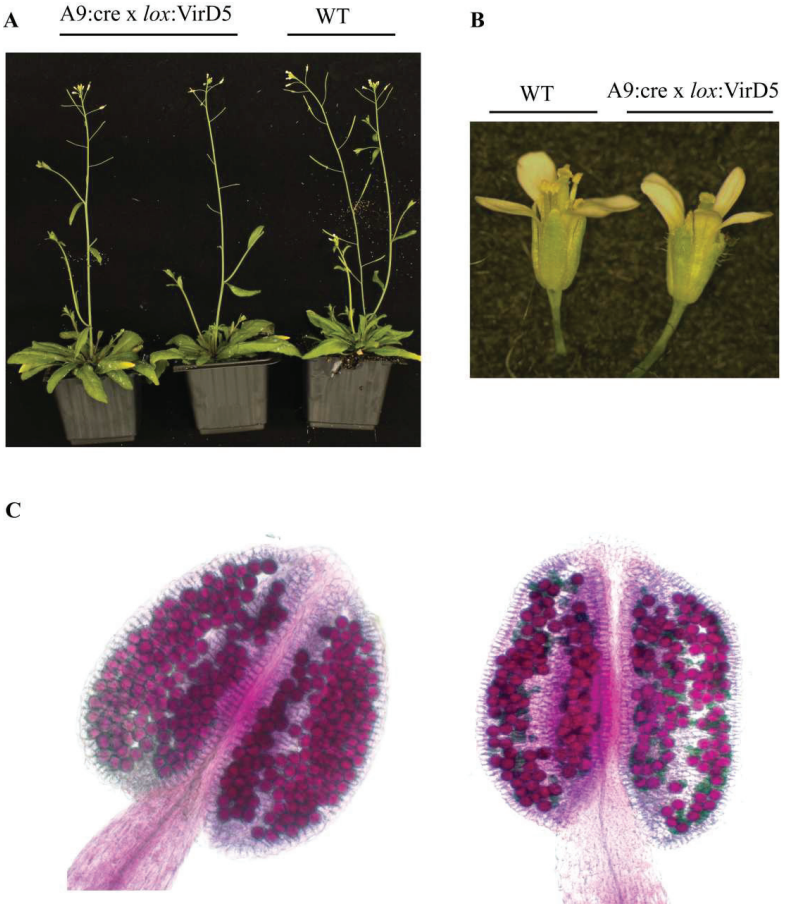


Figure 11. Inducible expression of VirD5 in *A. thaliana* pollen via a CRE/*lox*-GAL4/UAS double system leads to male sterile plants. (A) Silique development of VirD5 expressing plants in comparison to wild type plants. (B) Open flowers of wild type and VirD5 expressing plants. (C) Pollen viability staining in wild type (Left) and VirD5 expressing plants (Right). A9, tapetum specific promoter of *A. thaliana*. Cre, Cre recombinase.

cells. Transgenic plants expressing a low dose of VirD5 obtained by adding a low concentration of tamoxifen allowed us to follow root development. A much shorter and irregular root meristem was formed in the presence of tamoxifen (**Figure 6**). Besides a defective root meristem, we also saw the abundant formation of root hairs 4 days after germination, indicative of cell differentiation instead of division. DAPI staining of the cells from the irregular root meristem and the root hair cells often showed crashed or multiple nuclei (Data not shown). These data indicate that indeed VirD5 is targeted to the mitotic pathway in plant cells as well. To find out whether this may be due to errors in chromosome segregation, a GFP-LacI/LacO plant line was used to mark a single chromosome as a bright GFP dot in the nucleus, which can be visualized under the microscope to trace the marked chromosome. This powerful technique has been employed in several organisms, including

yeast, *Caenorhabditis elegans*, plants and mammalian cells (Meyer *et al*., 2013; Suijkerbuijk *et al*., 2012; Towbin *et al*., 2012). In our experiments aneuploidy was observed only in a few root cells (**Figure 7**). There are two possible interpretations: firstly, we only traced a single chromosome, while there are equal chances of chromosome mis-segregation for the remaining chromosomes in plant cells; secondly, we followed cell division after the induction of the expression of VirD5 only for 24 hours, which is not enough time for most of the cells to complete one division cycle due to a cell cycle arrest imposed by VirD5.

The toxic properties of VirD5 being causal of DNA damage and chromosome mis-segregation suggested that it might be used for cell ablation. We tested this by analyzing whether we could obtain male-sterile plants by local expression of VirD5.

Male sterile plants are frequently used for plant breeding. There are two main ways to acquire this trait such the classical cross breeding method and the genomic modification way which is achieved by expression of a cytotoxic gene specifically in pollen (Reprod, Zhan, and Cheung, 1996). The traditional procedure requires enormous time and efforts to hybrid plants, while the transgenic technique provides a quicker and more powerful way for generation of male infertility plants. In view of the toxic activity of VirD5 in plants, we used a double inducible system consisting of the Cre/*lox* in combination with the GAL4/UAS cassette to tightly control the expression of VirD5 in tapetum cells. Approximately half of the crossing plants exhibited manifest sterile pollen, while another half still displayed normal viable pollen. There are two possible factors that might explain this data; firstly the expression of VirD5 in tapetum cells may only cause defects of chromosome distribution in this layer of nutritive cells, whereas cells still have chance to gain correct number of chromosomes which might be enough to provide nutrient for pollen grains development; secondly VirD5 is transactivated by the 35S-driven GAL4-VP16 after the Cre recombinase mediated excision of *CRT1* in tapetum cells (**Figure 8**), the activity of the 35S promoter in tapetum cells might be too low to induce the expression of the GAL4-VP16 transcription activator. We also tested other pollen specific promoters such as DUO1, a gamete specific promoter from *A. thaliana* and LAT52 from tomato (Borg *et al*., 2011; Durbarry, Vizir, and Twell, 2005; Twell, Yamaguchi, and Mccormick, 1990). However, we could not observe the disrupted pollen grains in those plants crossed with VirD5 expressing plants. This technique paves the way for male sterile plants generation although there are still a few issues needs to be addressed including identifying promoters that are strongly active in the pollen to drive the expression of VirD5.

Earlier we found in yeast that the presence of VirD5 led to DNA damage and that VirD5 also targeted the Aurora kinases, essential mitotic regulators and consequently triggered host chromosome mis-segregation and aneuploidy. We confirmed the formation of DNA damage in human cells and the interaction of VirD5 with the Aurora kinases of human and plant cells. Aneuploidy, a hallmark of tumor formation, mainly arises from chromosome mis-segregation during mitosis (Holland and Cleveland, 2012). This indicates that VirD5 may play an important role in crown gall formation under natural conditions. Nevertheless, we found that the *Agrobacterium* mutant lacking *virD5* induced tumors on plant stems of equal size as that produced by the wild type strain (**Figure 8**). By using the more quantitative assay of tumor formation on roots we found, however, evidence that VirD5 is needed for optimal tumor development and that VirD5 affects tumor formation in a dose dependent way. Bacteria

introducing a T-DNA expressing VirD5 controlled by the 35S promoter induced extremely tiny plant tumors. Under natural conditions, only a very small amount of VirD5 is transferred into host cells during the infection process, and this dose might be enough to temporarily inhibit host cell division and arrest cells in a phase of the cell cycle beneficial for T-DNA insertion and/or tumor development. DNA damage may lead to DNA breaks which are the known entry points for T-DNA integration. Mammalian tumor cells show genome instability and are often aneuploid. This variation in genome content may generate cells with a variety of phenotypes of which those with the fastest growth become the most important in the development of tumor. The VirD5 protein may similarly generate such variety of transformed cells, leading to the evolution of plant cells that form a crown gall tumor. The results represent a completely novel strategy of a bacterial pathogen to modulate host cells.

Material and Methods

Floral dip assay

The first bolts of healthy growth *A. thaliana* were clipped to encourage proliferation of many second bolts (Clough and Bent, 1998; Logemann *et al*., 2006), after one week, the plants were ready for dipping. During this time, *Agrobacterium* Agl1 transformed with distinct binary vector was cultured in a large amount of liquid LC medium containing antibiotics overnight at 29 ^{o}C, and was suspended to OD_{600}=0.8 in 5% sucrose solution. Before dipping, silwet was added to the solution to a final contraction of 0.02% and mixed completely. After this, plants were dipped in the solution for several seconds and covered with a plastic bag overnight to maintain humidity, after several weeks' growth in green house, seeds were harvested and poured on MS media with antibiotics. The positive transgenic plants growing on selection media were soiled to pots for next generation growth.

Protoplast Transformation

The protoplast transformation was done following instructions according to Schirawski *et al* (2000) with slight modifications as follows. 10 μg of DNA for each plasmid was used in a single transformation. After adding the PEG solution we waited for 10 minutes before transferring the transformed cells to the plates containing protoplast medium. After transferring the cells for 30 minutes, the plates were sealed and incubated overnight at 25 °C in the dark. 18 hours later the transformed protoplasts were observed under the confocal microscope (Zeiss Observer).

Plasmids construction

Plant BIFC vectors were constructed as followings:VirD5 fragment was amplified with primers VirD5#15 and VirD5#24, PCR products digested with SalI and SpeI were cloned into SalI/SpeI fragment of both pRTL2-735 and pRTL2-736, resulting plasmids pRTL735:VirD5 and pRTL736:VirD5, respectively. Primers Aurora1FW/Aurora1REV, Aurora2FW/Aurora2REV and Aurora3FW/Aurora3REV were used to amplify Aurora1, Aurora2 and Aurora3 fragments, and PCR products digested with SalI and SpeI were subcloned into SalI/SpeI fragments of both pRTL2:735 and pRTL2:736, generating pRTL735:Aurora1/ pRTL736:Aurora1, pRTL735:Auror2/ pRTL736:Aurora2 and pRTL735:Aurora3/ pRTL736:Aurora3, respectively.

Pull-down plasmids were made as below: primers Aurora1gstF/ Aurora1gstR, Aurora2gstF/ Aurora2gstR and Aurora3gstF/Aurora3gstR were used for amplification of Aurora1, Aurora2 and Aurora3 fragments. PCR products digested with XmaI and XbaI were subcloned into XmaI and XbaI digested pGEX-KG vector, forming pGEX-KG: Aurora1, pGEX-KG: Aurora2 and pGEX-KG: Aurora3, respectively.

Binary vectors were constructed by following steps:VirD5 gene was amplified via VirD5#34 and VirD5#10-2 primers, PCR products cut with NotI were inserted into NotI fragment of pGPINTAM vector, producing pGPINTAM: VirD5 plasmid. 35S promoter fragment was cut off from pCAMBIA1302 with EcoRI/ XhoI and was ligated into EcoRI/SalI digested pCAMBIA1380, generating pCAMBIA1380:35S plasmid, *hygromycine* gene was removed from pCAMBIA1380:35S by cutting with XhoI and selfligation, forming pCAMBIA1380:35SΔ*hpt* vector. A cassette containing loxp-CRT1-loxp-GAL4VP16-UAS was cut off from pCB1 plasmid (a gift from Ben Scheres) (Heidstra, Welch, and Scheres, 2004) with HindIII and was inserted into HindIII lineated pCAMBIA1380:35SΔ*hp*, resulting in pCAMBIA1380:35S:cassette:Δ*hpt*, *virD5* together with NOS terminator fragment was cut off from pJET-VirD5-T by SpeI and was subcloned into SpeI digested pCAMBIA1380:35S:cassette:Δ*hpt and* pCAMBIA1380:35S, resulting in pCAMBIA1380:35S:cassette:VirD5:Δ*hpt* and pCAMBIA1380:35S:VirD5, respectively, and the former plasmid was renamed as pMDG1. Cre fragment was amplified using primers CreFW and CreREV, PCR products digested with NcoI/SpeI was ligated into NcoI/ SpeI fragment of pCAMBIA1390, forming pCAMBIA1390: Cre, the A9 promoter fragment amplified by primers A9FW/A9REV was cut with HindIII/NcoI for the subsequent insertion into HindIII/NcoI digested pCAMBIA1390: Cre, generating pCAMBIA1390: A9:Cre vector, renamed as pMDG2. Detail primers and plasmids information see **Table 1** and **2**, respectively.

BIFC

The pUG34VCn-VirD5 vector was transformed either with pUG35VNc-Spt4-1 or pUG35VNc-Spt4-2 into wild type (BY4743) yeast cells. Transformants were grown at 30 ºC on solid MY medium containing methionine to inhibit the expression of VirD5. After 3 days, colonies were transferred to MY liquid medium containing methionine. Overnight cultures washed twice with sterilized water were transferred into new flasks containing MY medium lacking methionine to induce the expression of VirD5. After induction for 1 hour, cells were harvested for BIFC signal visualization using a 63xoil objective on the Zeiss Imager confocal microscope (excitation, 514 nm; emission, 527 nm). Images were processed with ImageJ (ImageJ National Institutes of Health).

In vitro GST pull-down assay

Purified 6xHis-fused VirD5 protein was mixed with either empty GST tag or GST-Aurora1/GST-Aurora2/GST-Aurora3 bound to the Glutathione HiCap Matrix (Qiagen, 30900) for 2 hours at room temperature in 1xPBS buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2) containing 0.1% Triton X-100. After several washing steps with wash buffer (50 mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40), the samples were heated for 5 minutes at 100 °C in sample buffer, and subjected to SDS-PAGE

electrophoresis. The presence of the 6xHis-tagged VirD5 proteins was detected with Anti-6xHis HRP antibodies (Santa Cruz Biotechnology, sc-8036 HRP) by Western Blot analysis.

Pollen Alexander staining

Non-open or flower buds were collected and fixed in Carnoy's fixative (6 alcohol: 3 chloroform: 1 acetic acid) overnight at room temperature. Anthers from the fixed buds were dissected and put on slides, followed by adding drops of staining solution made in the following order (10 mL 95% ethanol, 1 mL 1% malachite green, 50 mL distilled water, 25 mL glycerol, 5 mL 1% acid fuchsin, 0.5 mL 1% orange G, 4 mL glacial acetic acid and 4.5 mL distilled water) for 1 hour at 55 ^{o}C. Samples were washed by drops of pure water before putting cover slides on. The stained pollen grains were observed under the DIC microscopy with 20x and 40x objectives.

Tumor assay

Four-week old *Nicotiana glauca* seedlings were inoculated with 20 μl of *Agrobacterium tumefaciens* suspended in 0.9% NaCl to an OD_{600} of 1. Tumors were photographed after 3 weeks.

Root transformation

Root segments from *Arabidopsis thaliana* seedlings were infected with *Agrobacterium* strains at a final concentration of OD_{600}=0.1 in B5 medium for 2 minutes and were subsequently incubated on hormone-free B5 medium for 2 days. After incubation, roots were washed in B5 liquid medium and transferred to fresh hormone-free B5 medium containing 100 mg/L timentin. Calli were scored 3-4 weeks after incubation.

Acknowledgements

We thank Marjori Matzke for the LacO/GFP-LacI plant lines, Ben Scheres for the plasmid. We would like to thank Gerda Lamers for invalubale technical support on microsopy. This work was supported by the China Scholarship Council (CSC).

Table 1. Primers used in this study.

Name	Sequences (5'-3')	Restriction site (underlined)
Vir5#1	CCGCCCCGGGGATGACAGGAAAG	XmaI
VirD5#10-2	AAAGCGGCCGCTCAGCGTTTAAACGC	NotI
VirD5#15	GCGTCGACAATGACAGGAAAGTCG	SalI
VirD5#21	CCATCGATATGACAGGAAAGTCG	ClaI
VirD5#21-2	CCCCCCGGGTCAGCGTTTAAAC	XmaI
VirD5#24	GGACTAGTTCAGCGTTTAAACGCT	SpeI
VirD5#30	GCTCTAGATCAGCGTTTAAACGCT	XbaI
VirD5#34	AAAGCGGCCGCAACAGGCTGATGCCTCGTTTG	NotI
VirD5#36-2REV	CCATCGATACTAGTCCTCGACTCGGTACCCCCTCGACAC	ClaI and SpeI
VirD5#63	ACGCGTCGACGGAGATATACCATGGGC	SalI
VirD5#38	GGACTAGTATGACAGGAAAGTCGAAAGTTCAC	SpeI
Aurora1FW	ACGCGTCGACAATGGCGATCCCTACGGAG	SalI
Aurora1REV	GGACTAGTTTAAACTCTGTAGATTCC	SpeI
Aurora2FW	ACGCGTCGACAATGGGGATTTCTACAGAG	SalI
Aurora2REV	GGACTAGTTCATCCTCTGTAAAGGCC	SpeI
Aurora3FW	ACGCGTCGACAATGAGTAAGAAATCGACA	SalI
Aurora3REV	GGACTAGTTCAAATATCAATTGAGGC	SpeI
Aurora1gstF	CCCCCCGGGAATGGCGATCCCTACGGAGAC	XmaI
Aurora1gstR	GCTCTAGATTAAACTCTGTAGATTCCAG	Xbal1
Aurora2gstF	CCCCCCGGGAATGGGGATTTCTACAGAGAC	XmaI
Aurora2gstR	GCTCTAGATCATCCTCTGTAAAGGCCTG	XbaI
Aurora3gstF	CCCCCCGGGAATGAGTAAGAAATCGACAGA	XmaI
Aurora3gstR	GCTCTAGATCAAATATCAATTGAGGCAC	XbaI
CreFW	GCCCATGGATGTCCAATTTACTGACCGTA	NcoI
CreREV	GGACTAGTCTAATCGCCATCTTCCAG	SpeI
A9FW	CCCAAGCTTGGGGAAATAGATTTTCTCTACTG	HindIII
A9REV	GCCCATGGTCTAATTAGATACTATATTGTTTGTAC	NcoI
P1	CATGTTGTAGGTGACTCATGGGAAC	
P2	TGCGCAGCCTGAATGGCGCCCGCTC	
P3	GCGATGGTCTGCAAAGTGAATCGC	
P4	ATGAGTAGAGAGAGTCGTCTGTCTC	
pSpt4_1F	GGACTAGTCATGGGAGAAGCGCCTGCCCAGATTCCG	SpeI
pSpt4_1R	ACGCGTCGACGAATACGTTTGGGTGGAACGTACTGCACCCG	SalI
pSpt4_2F	GGACTAGTCATGGGAAGCGCACCAGCTCAGATTCCG	SpeI
pSpt4_2R	ACGCGTCGACGGGGAGTGGCTCTGAGACAGCAAGTGTG	SalI

Table 2. Plasmids used in this study.

Name	Descriptions	Sources/references
pGPINTAMNOTI	Binary vector with a tamoxifen inducible promoter.	(Friml *et al*., 2004)
pGPINTAMNOTI-VirD5	VirD5 (NotI) was inserted into pGPINTAMNOTI.	This study
pRTL735	Plant BIFC vector with an N-terminal fusion with the C-terminus of YFP.	(Bracha-Drori *et al*., 2004)
pRTL735-VirD5	VirD5 (SalI/SpeI) was inserted into pRTL735.	This study
pRTL735-Aurora 1	Aurora 1 (SalI/SpeI) was inserted into pRTL735.	This study
pRTL735-Aurora 2	Aurora 2 (SalI/SpeI) was inserted into pRTL735.	This study
pRTL735-Aurora 3	Aurora 3 (SalI/SpeI) was inserted into pRTL735.	This study
pRTL736	Plant BIFC vector with an N-terminal fusion with the N-terminus of YFP.	(Bracha-Drori *et al*., 2004)
pRTL736-VirD5	VirD5 (SalI/SpeI) was inserted into pRTL736.	This study
pRTL736-Aurora 1	Aurora 1 (SalI/SpeI) was inserted into pRTL736.	This study
pRTL736-Aurora 2	Aurora 2 (SalI/SpeI) was inserted into pRTL736.	This study
pRTL736-Aurora 3	Aurora 3 (SalI/SpeI) was inserted into pRTL736.	This study
34VCn	Single-copy plasmid with an N-terminal fusion with the C-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
34VCn-VirD5	VirD5 (SpeI/ SalI) was inserted into 34VCn.	This study
35VNc	Single-copy plasmid with an C-terminal fusion with the N-terminal Venus part driven by the MET25 promoter.	(Sakalis, 2013)
35VNc-pSt4-1	Spt4-1 (SpeI/SalI) was inserted into 35VNc.	This study
35VNc-pSt4-2	Spt4-2 (SpeI/SalI) was inserted into 35VNc.	This study
pET-16H	pBR322 base plasmid with an N-terminal10xHis tag under the control of the T7 promoter.	Novagen
pET-16H-VirD5	VirD5 (ClaI-XmaI) was inserted into pET-16H.	This study
pGEX-KG	pMB1 based plasmid with an N-terminal GST tag under the control of the TAC promoter.	(Guan and Dixon, 1991)
pGEX-KG-Aurora 1	Aurora 1 (XmaI/XbaI) was inserted into pGEX-KG.	This study
pGEX-KG-Aurora 2	Aurora 2 (XmaI/XbaI) was inserted into pGEX-KG.	This study
pGEX-KG-Aurora 3	Aurora 3 (XmaI/XbaI) was inserted into pGEX-KG.	This study
pCAMBIA1380-35S-VirD5t	VirD5 was inserted into pCAMBIA1380-35S	This study
pCAMBIA1380-35S (ΔH)-lox-CRT1-lox-GAL4VP16-UAS-VirD5t	lox-CRT1-lox-GAL4VP16-UAS-VirD5t was inserted into pCAMBIA1380-35S.	This study
pCAMBIA1390-A9-Cre	A9-Cre was inserted into pCAMBIA1390.	This study
pBBR6-VirD5	VirD5 (SalI/ XmaI) was inserted into pBBR6.	This study

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Chapter 5

The VirD5 protein interferes with mitosis in human cells

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Abstract

Faithful chromosome segregation is a prerequisite for the maintenance of genome integrity during the cell cycle. Defects in the chromosome segregation machinery cause chromosome instability (CIN), and may lead to tumor formation. Previously, we found that the *Agrobacterium tumefaciens* virulence protein VirD5 interacts with the sole Aurora kinase of yeast called Ipl1 and causes chromosome mis-segregation. Here we expressed VirD5 in human osteosarcoma cells (U2OS), and found that it can also interact with the three mammalian Aurora kinases, AurkA, AurkB and AurkC which are essential for cell cycle regulation and faithful chromosome segregation in mammalian cells. The VirD5 protein colocalizes with PCNA in interphase and thus may then be present at replication sites. During mitosis, however, VirD5 is present at the centrosomes. Expression of VirD5 in human cells causes incorrect chromosome segregation, chromosome fragmentation and also micronuclei (MN) formation during mitosis. As a result many cells become apoptotic. The formation of micronuclei is a hallmark of tumorigenesis, which may underlie the role of VirD5 in plant tumor formation. The results from this study are in line with our previous data and indicate that VirD5 affects the strongly conserved Aurora kinases in yeast, plant and mammalian cells subverting the essential pathway of host cell division during infection.

Introduction

Accurate chromosome segregation requires the bipolar attachment of the sister chromatids to spindle microtubules (MT) nucleated from opposite spindle poles during mitosis. Kinetochore proteins and regulatory kinases together with the checkpoint signaling pathway ensure a high fidelity of chromosome segregation. The kinetochore, a protein complex, assembles on the centromeric DNA and attaches to spindle microtubules to mediate chromosome movement to daughter cells during cell division. Improper chromosome-microtubule attachment such as merotelic (spindle microtubules from two poles attach to a single kinetochore) and syntelic (spindle microtubules from the same pole attach to both sister kinetochores) stimulates the spindle checkpoint until the wrong attachments have been corrected by the cell cycle regulatory proteins (Maiato *et al*., 2004). If these erroneous bindings are not corrected, they can cause chromosome mis-segregation, aneuploidy and micronuclei formation (Thompson and Compton, 2011).

Aneuploidy is the state that cells contain uneven numbers of chromosomes and is to a large extent associated with developmental diseases and cancer (Pariente, 2012). A micronucleus, an independent nucleus-like compartment, may be formed from lagging chromosomes or chromosome bridges after mitosis (Shimura *et al*., 1999). Both aneuploidy and micronuclei are hallmarks of cancer.

In budding yeast, the Ipl1/Aurora B kinase plays an essential role in bipolar chromosome attachment and correct chromosome segregation by destabilizing erroneous attachments between kinetochore and spindle microtubule. It accomplishes this by phosphorylating a series of substrates including the microtubule binding protein Dam1 and the outer kinetochore protein Ndc80 (Lampert *et al*., 2013). There are three Aurora kinases in mammalian cells, AurkA, AurkB and AurkC, which like Ipl1 contain a core C-terminal catalytic domain required for its kinase activity, and an N-terminal regulatory domain which mostly interacts with the distinct substrates for the subsequent phosphorylation by the C-terminal kinase domain. They are distributed to distinct sites in the cell and therefore fulfill different functions during the cell cycle (Fu *et al*., 2007). Aurora A is mainly involved in centrosome formation and spindle assembly; it also can catalyze detachment of incorrect microtubule-kinetochore attachments by phosphorylating microtubule-associated proteins and kinetochore proteins of chromosomes in the vicinity of the spindle poles (Ye *et al*., 2015). Inhibition of Aurora kinase A leads to the centrosomes abnormality and chromosome mis-segregation, causing aneuploidy and micronuclei formation. Its overexpression also stimulates chromosome instability (Zhu *et al*., 2005).

The Aurora B kinase localizes to the centromeres from prophase to metaphase and relocates to the central spindle midzone during anaphase and eventually accumulates in the midbody until cytokinesis is complete (Biggins *et al*., 1999). It forms a complex with three other proteins called INCENP, Survivin and Borealin, forming the Chromosomal Passenger Complex (CPC) to aid bi-polar chromosome attachment by destabilizing improper kinetochore-microtubule attachments by phosphorylating kinetochore proteins including the core outer kinetochore protein Ndc80 (Akiyoshi *et al*., 2009). Inhibition or overexpression of the Aurora B kinase leads to chromosome mis-segregation and aneuploidy formation, and consequently causes tumorigenesis (Takeshita *et al*., 2013; Vader and Lens, 2008). Aurora C shares high homology with the other two Aurora kinases. Although its biological function has

not been completely clarified yet, Aurora C can substitute for Aurora B kinase activity, suggesting that it also may be (redundantly) involved in the cell division process (Carmena and Earnshaw, 2003; Fu *et al*., 2007).

A. tumefaciens, a Gram-negative soil bacterium, is capable of infecting a large number of dicotyledonous plant species, causing crown gall disease. During infection, a segment of DNA called T-DNA is transferred from *Agrobacterium* into plant cells and integrated into the host genome. Besides, several virulence proteins including VirE2, VirE3, VirF and VirD5 are transferred into the host to assist in the transformation process (Vergunst *et al*., 2000).

In the previous chapters, it was shown that the *A. tumefaciens* virulence protein VirD5 interacted with yeast and plant Aurora kinases and stimulated their kinase activities. The increased kinase activities are known to destabilize the attachment between microtubule and kinetochore and to cause chromosome mis-segregation during mitosis. Cells expressing VirD5 were indeed arrested in M phase and ultimately chromosome mis-segregation and aneuploidy was observed. In view of the high sequence homology among Aurora kinases from different eukaryotes and their important roles in regulating cell mitosis, here we investigated whether VirD5 could also similarly affect human cells. We found that VirD5 indeed could interact with the three mammalian Aurora kinases, and that expression of VirD5 in human cells led to chromosome mis-segregation and micronuclei formation and apoptosis.

Results

Aurora kinases are highly conserved in eukaryotes

In the previous chapter, it was shown that VirD5 interacts with the yeast Aurora kinase Ipl1 with as a consequence chromosome mis-segregation. Aurora kinases have several vital roles during cell mitosis, and consist of two functional domains (Fu *et al*., 2007): a variable N-terminal regulatory domain and a conserved C-terminal catalytic domain. To find out to which of these parts VirD5 binds, we did an *in vitro* pull-down assay using either the N-terminal or the C-terminal part of Ipl1. As can be seen in **Figure 1A** VirD5 exclusively interacted with the catalytic part of the Ipl1 kinase. This is the part that is conserved between Aurora kinases from different organisms as can be seen in the sequence alignment of the C-termini of Aurora kinases from human, yeast and plant cells (**Figure 1B**).

VirD5 interacts with human Aurora kinases

Mammalian cells contain three Aurora kinases: AurkA, AurkB and AurkC. In view of the high sequence homology of the catalytic domains of different Aurora kinases, we wondered whether VirD5 can interact with these mammalian Aurora proteins as well. We first tested this hypothesis in budding yeast via a Bimolecular Fluorescent Complementation (BIFC) assay (Kerppola, 2006). Fluorescent punctate foci, representing the reconstituted signal were seen in the yeast cells transformed with plasmids encoding both VirD5 and Aurora proteins, but not in the cells harboring either the plasmid encoding VirD5 or that encoding the Aurora proteins alone (**Figure 2A**). In order to confirm these interactions, we performed an *in vitro* pull-down assay. VirD5 fused with a 6xHis tag at its N-terminus and AurkA, AurkB and AurkC fused in frame with glutathione-S-transferase (GST) were expressed and purified from *E.coli*. Purified His-tagged VirD5 was incubated with either empty GST or GST-tagged Aurora kinases *in vitro* for 2 hours at room temperature. By pulldown experiments it was

found that VirD5 had a strong physical interaction with the three GST-tagged Aurora proteins, but no binding was observed to the empty GST (**Figure 2B**). The interaction of VirD5 with AurkB was further confirmed in a co-immunoprecipitation assay from human osteosarcoma cells that had been co-transfected with constructs expressing myc-tagged Aurora B protein and either mCherry-tagged VirD5 or as a negative control the mCherry-tagged truncated VirD5N202 protein, which does not bind to Aurora kinases (**Figure 2C**).

VirD5 dynamics during the human cell cycle

The interactions with the essential human mitotic Aurora kinases indicate that VirD5 might affect the mammalian cell cycle. We first determined the sublocation of the VirD5 protein in human cells. mCherry-tagged VirD5 under the control of a tetracycline-inducible promoter was transfected into U2OS-TR cells which continuously express TetR to repress the expression of VirD5. The N-terminal 202aa of VirD5 (VirD5N202) which is nontoxic to yeast cells (**Chapter 2**) fused with mCherry was used as a control. After induction with doxycycline for 24 hours, we observed bright fluorescent punctate foci in the nuclei of interphase cells expressing VirD5 (**Figure 3**, a-c) and co-localization with the centrosomes in mitotic cells (**Figure 3**, d-f and **Figure 5A**). In contrast a diffuse cytosolic and nuclear sublocation signal was seen in control cells expressing VirD5N202 (**Figure 3**, g-i). Interestingly, 60% of the transfected cells expressing VirD5 exhibited the presence of micronuclei (**Figure 3**, a-c), which was only observed in 5% of the control cells. This represents a first indication that the expression of VirD5 also affects chromosome segregation in human cells.

In eukaryotes, DNA replication occurs in discrete replication foci in the nucleus during S phase, and is mediated by a complex replication machinery which includes proliferating cell nuclear antigen (PCNA) (Bravo and Macdonald-Bravo, 1987). To determine whether the punctate foci of VirD5 seen in the nuclei of interphase cells might be associated with the DNA replication machinery, we co-expressed the Flag-tagged VirD5 protein with GFP-PCNA (Leonhardt and Rahn, 2000) in U2OS cells for 24 hours and analyzed the position of VirD5 by immunofluorescence with anti-Flag antibody, in comparison to that of PCNA by GFP fluorescence. As shown in **Figure 4**, VirD5 colocalized completely with the PCNA punctate foci in the nucleus during early S phase and partially in late S phase, suggesting that VirD5 might target the DNA replication pathway in S phase.

The centrosome is a small organelle that consists of two centrioles surrounded by pericentriolar material (PCM). The main function of the centrosomes is to serve as microtubule organization center (MTOC) and especially to generate the bi-polar spindle of microtubules, which is a prerequisite for faithful chromosome segregation over the daughter cells during mitosis (Pihan *et al.*, 2003; Tanenbaum and Medema, 2010). The centrosome is duplicated in the G1/S stage and the duplicated centrosomes are separated late in the G2 phase (Hinchcliffe and Sluder, 2002). There is evidence that the Aurora A kinase is localized at the centrosome to facilitate centrosome maturation and separation, and that ectopic expression of Aurora A causes tumorigenesis (Smith *et al.*, 2005). The accumulation of VirD5 at the centrosomes in human cells suggests that it may interact with the Aurora A kinase *in vivo* (**Figure 2A** and **B**). In order to find out when VirD5 moved to the centrosomes

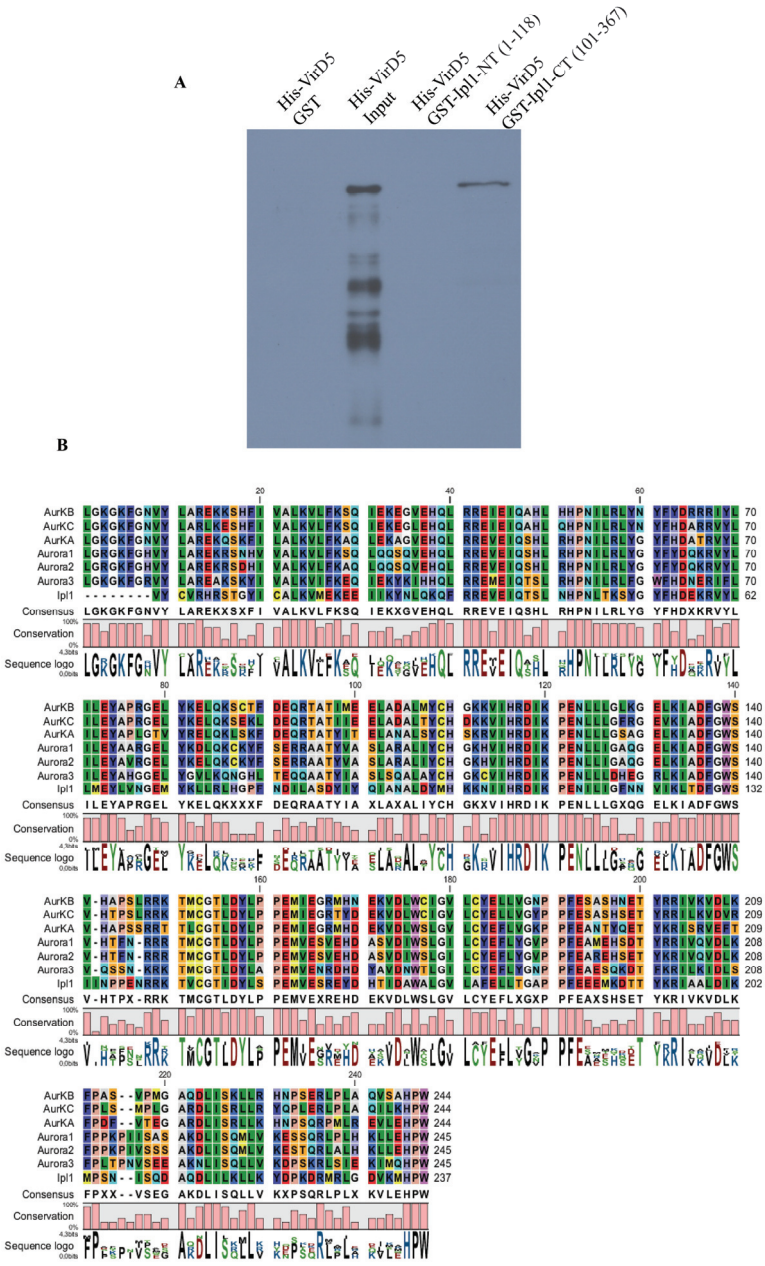

Figure 1. VirD5 physically interacts with the conserved catalytic domain of Ipl1/Aurora B kinase. (A) His-tagged VirD5 purified from *E.coli* was incubated with either empty GST or GST-Ipl1-NT or GST-Ipl1-CT; after washing, the presence of His-VirD5 was detected by anti-His antibody. The experiment was repeated three times. (B) The catalytic domain sequences of Aurora kinases from yeast, plant and human cells were aligned using CLC software. NT and CT represent the N-terminus (1-118aa) and the C-terminus (101-367aa) of Ipl1, respectively.

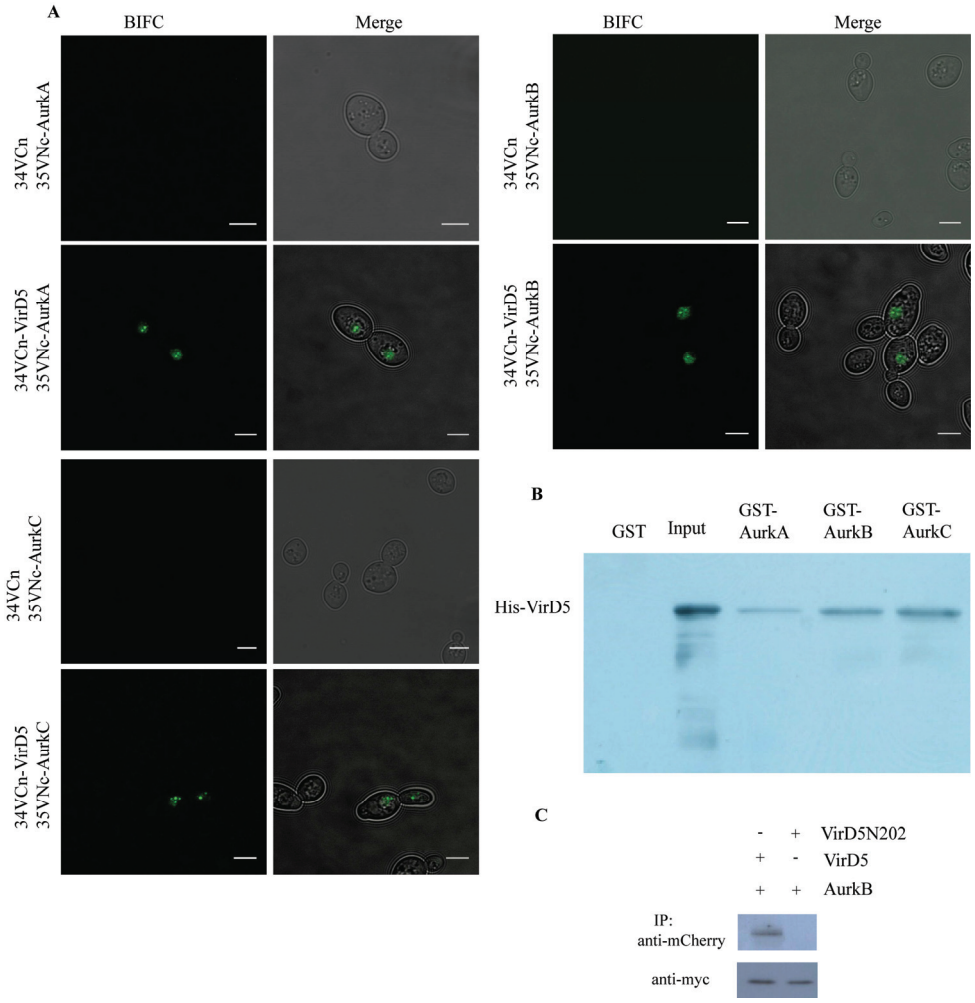

Figure 2. VirD5 physically interacts with the three Aurora kinases from human cells. (A) Yeast cells transformed with 35VNc-based BIFC vectors containing AurkA, B or C together with 34VCn-based either empty or VirD5 containing vectors. 35VNc: the N-terminus of YFP (VN173) fused with the C-terminus of testing proteins. 34VCn: the C-terminus of YFP (VC173) fused with the N-terminus of testing proteins. Scale bar, 5μm. (B) His-tagged VirD5 purified from *E.coli* was incubated with either empty GST or GST-tagged Aurora kinases. After washing out the non-specific binding proteins, the presence of His-VirD5 was detected by western blot using anti-His antibody. (C) Co-immunoprecipitation of myc-tagged AurkB with mCherry fused VirD5 or mCherry fused VirD5N202 proteins from U2OS cells.

during the cell cycle, we transfected U2OS-TR cells with a construct encoding GFP-VirD5 driven by the tetracycline-inducible promoter, grew these cells in the presence of doxycycline and then the microtubules were visualized by immunostaining using rat anti-tubulin antibody. The result illustrated that VirD5 was present at the centrosomes exclusively in M phase (**Figure 5A**, g-i), but localized at DNA replication foci during interphase (**Figure 5A**, a-f),

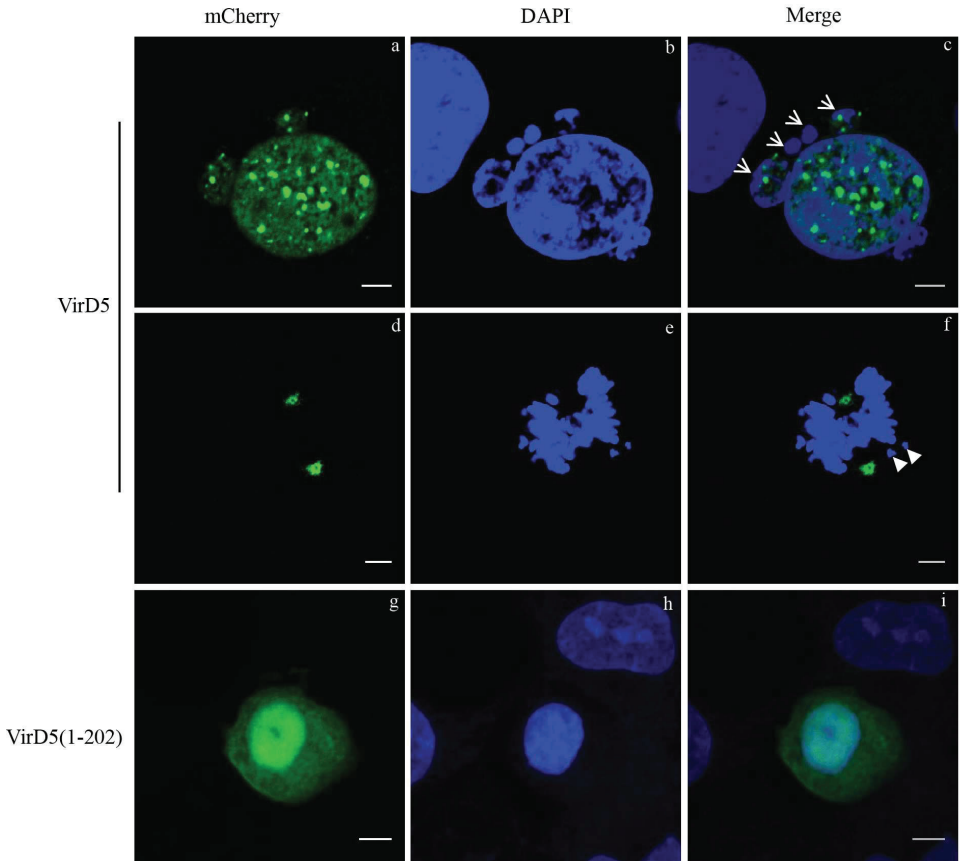

Figure 3. The VirD5 protein is present at punctate foci in the nuclei of U2OS cells in interphase, but at the centrosomes in mitosis. U2OS-TR cells transfected with plasmids encoding mCherry-VirD5 (a-f) or mCherry-VirD5 (1-202) (g-i) as the negative control. The localization of mCherry-tagged proteins was detected by immunostaining with anti-mCherry antibody. The nuclei were stained with DAPI (4', 6-diamidino-2-phenylindole). Arrows point to micronuclei; arrow heads point to free unaligned chromosomes. Scale bar, 5μm.

suggesting that VirD5 then has a higher affinity for components of the DNA replication machinery than for Aurora A kinase.

VirD5 induces chromosome mis-segregation and micronuclei formation

More than 60% of the U2OS-TR cells in interphase expressing VirD5 showed micronuclei (MN) (**Figure 3**, a-c). MN, a hallmark of chromosome segregation errors mainly originate from lagging chromosomes that fail to be faithfully separated into daughter cells during telophase (Savage, 2011; Unit, 1988). There are several mechanisms that can cause lagging chromosomes including the presence of multi-polar mitotic spindles, which are the consequence of the formation of multiple centrosomes (Kline-Smith and Walczak, 2004; Holland and Cleveland, 2012a; Sato *et al*., 2001), and incorrect chromosome attachments to spindle microtubules during mitosis (Holland and Cleveland, 2012b).

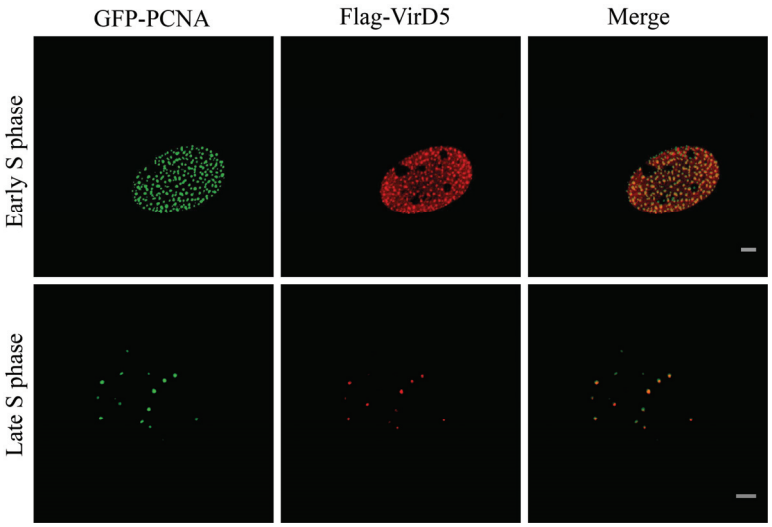

Figure 4. Location of VirD5 in punctate foci in the nucleus of interphase cells and colocalization with DNA replication protein PCNA. U2OS cells were transfected with plasmids encoding Flag-tagged VirD5 and GFP-PCNA and 24 hours after transfection, cells were fixed for detection of Flag-VirD5 by immunofluorescence using anti-Flag antibody and of GFP-PCNA. Yellow foci represent the colocalization of both proteins. Scale bar, 5μm.

As VirD5 accumulated at the centrosomes in the mitotic stage, we first tested whether targeting the centrosomal structure by VirD5 might affect centrosome duplication. To test this, we transfected U2OS-TR cells with a plasmid encoding mCherry-VirD5 or mCherry-VirD5N202 as a control. 24 hours after transfection, cells were switched to media containing doxycycline to induce the expression of the mCherry-tagged proteins and nocodazole to cause cell cycle arrest in metaphase. Centrosomes were detected in these cells by immunostaining with anti-γ-tubulin antibody. Both the cells expressing the intact VirD5 protein and those expressing the control protein mainly showed two centrosomes in metaphase cells (**Figure 5B**). Apparently, centrosome duplication occurs normally in cells expressing VirD5. This is in line with our finding that VirD5 accumulates at the centrosomes exclusively in M phase (**Figure 5A**) after the centrosomes have been duplicated.

Our *in vitro* experiments showed that VirD5 can physically interact not only with Aurora A, but also with the Aurora B and Aurora C proteins (**Figure 2A** and **B**), which play essential roles in the accurate segregation of chromosomes. To gain further information about the effects of VirD5 expression in U2OS-TR cells, cells stably transformed with the gene encoding mCherry-VirD5 under the control of the tetracycline-inducible promoter were treated for 16 hours with either doxycycline or DMSO (as a negative control). The chromosomes of mitotic cells were stained and examined. It turned out that 70% of cells expressing VirD5 showed either unaligned or lagging chromosomes (**Figure 6**, g-o), whereas in contrast, only 20% of control cells exhibited this phenotype. This data clearly indicates that VirD5 induces chromosome mis-segregation in human cells, which is in line with our previous results from both yeast and plant cells.

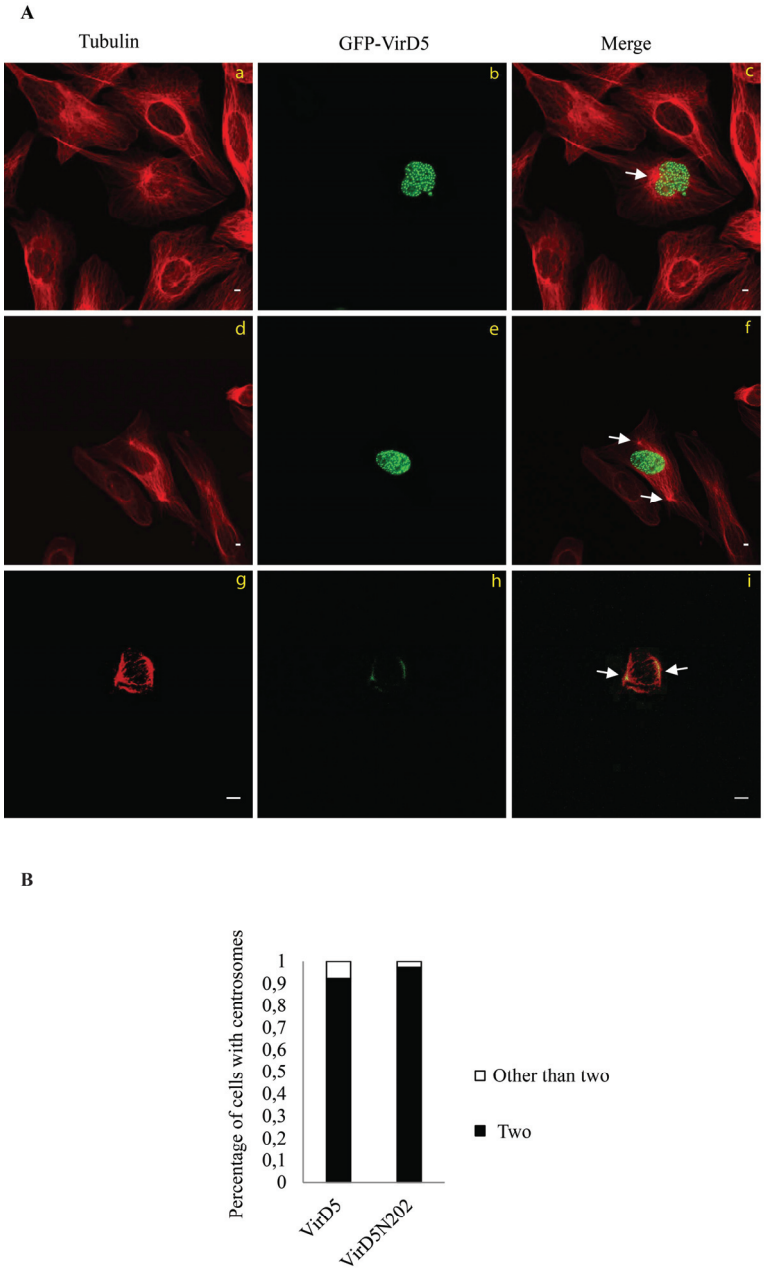

Figure 5. Centrosome duplication occurs normally in cells expressing VirD5(A) U2OS-TR cells were transfected with a plasmid encoding GFP-VirD5 driven by the tetracycline inducible promoter, and after induction with doxycycline cells were fixed and microtubules were visualized by immunostaining with anti-Tubulin antibody. Cells from interphase (a-f) and M (g-i) phases were selected. Arrows point to the centrosomes. Scale bar, 5μm. (B) U2OS-TR cells expressing either mCherry-VirD5 or mCherry-VirD5N202, were fixed and the centrosomes were visualized by immunostaining with anti-γ-tubulin antibody. 100 mitotic cells were counted in each independent experiment. This experiment was repeated three times.

Lagging chromosomes may originate either from broken chromosomes and especially from chromosome fragments without centromere or from whole chromosomes that failed to be properly segregated due to an incorrect microtubule-kinetochore attachment (Fenech *et al.*, 2011). To gain insight into the status of the lagging chromosomes that were seen after VirD5 expression, we used Ndc80/Hec1 as the kinetochore marker. It turned out that 30-45% of these lagging chromosomes were lacking a kinetochore and by inference a centromere (**Figure 6**, j-l), whereas the remainder had a centromere (**Figure 6**, m-o). This indicates that the majority of the chromosomes mis-segregated due to the presence of VirD5 are a consequence of improper attachments between kinetochore and microtubule. Besides, the presence of small chromosomal fragments in virD5 expressing cells (**Figure 6**, i, l, o) suggests that VirD5 may cause chromosome breakage in human cells.

VirD5 triggers DNA damage

In order to find out whether VirD5 may cause directly or indirectly DNA damage and chromosome instability in cells, we transfected U2OS cells with a plasmid encoding mCherry-VirD5 (or as a control mCherry-VirD5N202) driven by the constitutively active CMV promoter. After 24 hours of transient expression, cells were fixed and foci of DNA damage were visualized by immunostaining with anti-γ-H2AX antibody. Only 20% of cells transfected with the control plasmid showed DNA damage foci, whereas over 60% of cells expressing VirD5 displayed γ-H2AX foci in the nuclei (**Figure 7**). Subsequently, we analyzed whether this DNA damage could alter the chromosomal structure. To this end, chromosome morphologies were surveyed in U2OS-TR-mCherry-VirD5 cells that had been grown in the presence or absence of doxycycline. As shown in **Figure 8**, the ectopic expression of VirD5 induced chromosome fragmentation in about 50% of mitotic cells. These results are in line with the data from yeast cells expressing VirD5, where chromosome fragmentation was seen after chromosome analysis on CHEF gels (**Chapter 2**). These results suggest that the increased chromosome mis-segregation and micronuclei formation brought about by VirD5 may at least partially be due to DNA damage and chromosome fragmentation.

Apoptosis is induced by the presence of VirD5

The presence of DNA lesions may activate the DNA damage checkpoint and thus lead to cell cycle arrest to allow DNA repair before cell division. In higher eukaryotes it may also trigger the apoptotic process to eliminate heavily damaged cells from the organism and so prevent tumorigenesis (Norbury and Zhivotovsky, 2004). In order to test whether expression of VirD5 could lead to apoptosis, we performed a long time live-imaging study of U2OS-TR cells cotransfected with plasmids encoding mCherry-VirD5 under the control of the doxycycline-inducible promoter and GFP-Tubulin. Cells expressing both GFP-Tubulin and mCherry-VirD5 were followed over time as well as neighboring cells lacking the mCherry signal (negative control). As shown in **Figure 9**, VirD5-expressing cells eventually underwent cell death. This indicated that the DNA damage and chromosome fragmentation induced by VirD5 leads to permanent cell arrest or to apoptosis.

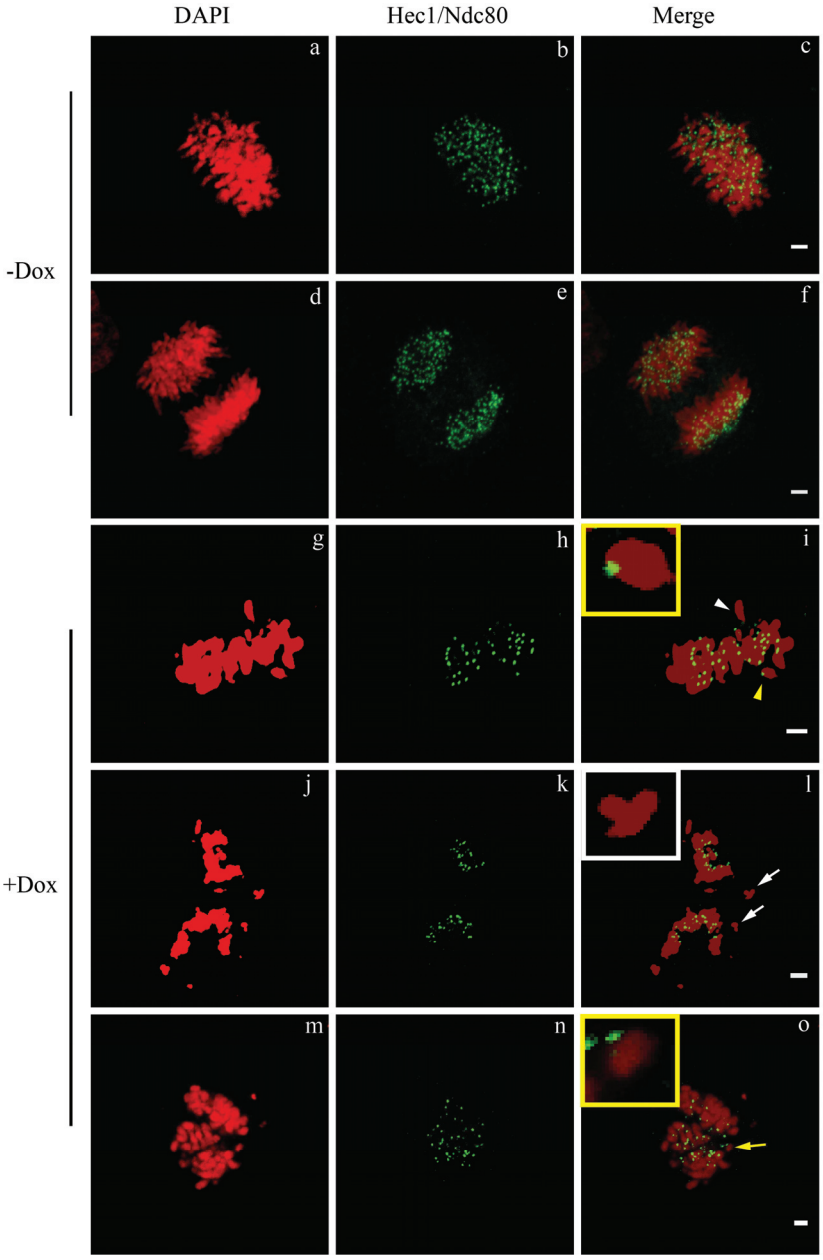

Figure 6. VirD5 causes chromosome mis-segregation. A U2OS-TR-mCherry-VirD5 stable cell line was treated with either doxycycline or DMSO for 16 hours; cells were fixed for kinetochore detection by immunofluorescence via anti-Hec1 antibody (green). DNA was stained with DAPI (red). White arrows represent acentric lagging chromosomes; yellow arrow represents a lagging chromosome with a centromere; white arrowhead points to an unaligned acentric chromosome; yellow arrowhead points to an unaligned chromosome with a centromere. 50 cells were observed. Scale bar, 5μm.

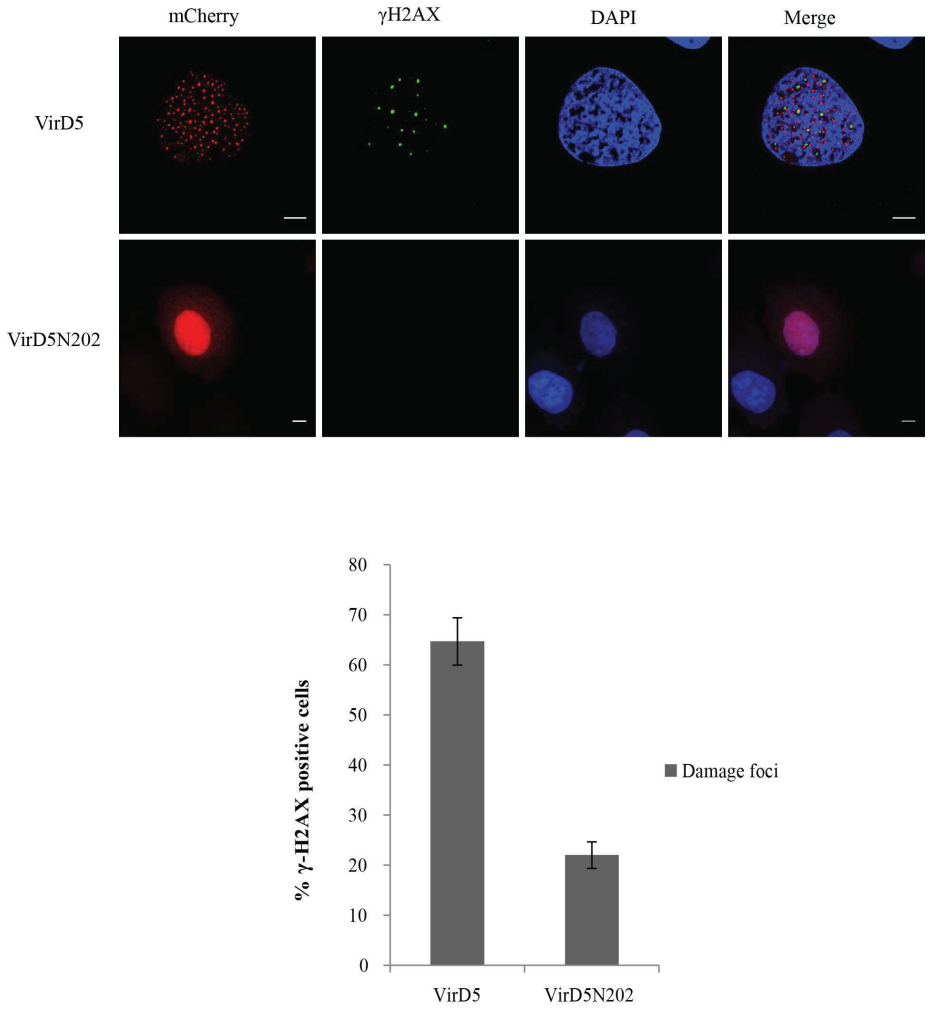

Figure 7. VirD5 triggers DNA damage. U2OS cells transfected with plasmids encoding either mCherry-VirD5 or mCherry-VirD5N202 as a control for 24 hours were fixed and the DNA damage foci were detected by immunofluorescence via anti-γ-H2AX antibody. DNA was stained with DAPI. VirD5N202, the N-terminal non-toxic 202aa of VirD5. 100 cells were observed. Scale bar, 5μm. Error bars represent the mean ± SD from three independent experiments.

Discussion

Accurate chromosome segregation during mitosis is essential for maintaining the genetic material. Errors in chromosome distribution can lead to aneuploidy and micronuclei, all hallmarks of tumor cells. Faithful chromosome segregation is controlled by Aurora kinases in eukaryotes. Previously, we have found that the *A. tumefaciens* virulence protein VirD5 interacts with Aurora kinases from both plant and yeast cells (**Chapter 2**, **3** and **4**). The physical interaction with Ipl1/Aurora B in yeast stimulated its kinase activity and led to chromosome mis-segregation (**Chapter 3**). Aurora kinases share a highly homologous C-

terminal kinase domain, but have a variable N-terminal regulatory domain. By an *in vitro* pull-down assay we found that VirD5 interacted with the highly conserved C-terminal catalytic domain of yeast Aurora B/Ipl1 kinase (**Figure 1**). This result suggested that VirD5 might also interact with human Aurora kinases.

In mammalian cells, there are three Aurora kinases involved in cell cycle regulation (Carmena and Earnshaw, 2003; Lens, Voest, and Medema, 2010). In yeast VirD5 is located at the centromeres/kinetochores and interacts with the yeast Aurora kinase Ipl1 (**Chapter 3**), which is also present at the centromeres from prophase up to metaphase (Biggins *et al*., 1999). However, in human cells we found that VirD5 is located at the centrosomes during mitosis (**Figure 3** and **5A**). Therefore, in human cells VirD5 probably does not exert its effects through an interaction with Aurora B, which like Ipl1, is present at the centromeres during early mitosis up to metaphase, and then transfers to spindle midzone and concentrates at the midbody during telophase and cytokinesis (Fu *et al*., 2007) and is involved in the correction of erroneous kinetochore-microtubule attachments (Saurin *et al*., 2011). But in view of its sublocation in human cells VirD5 might *in vivo* rather interact with human Aurora A, which is present at the centrosome and plays essential roles in centrosome maturation and duplication, spindle assembly and chromosome segregation (Andrews *et al*., 2003). In a recent paper it was shown that Aurora A contributes from a centrosomal localization to the correction of improper kinetochore-microtubule attachments by opposing the stabilizing effect of polar ejection forces (PEFs) (Cane *et al,* 2013). As such Aurora A also contributes to the prevention of chromosome mis-segregation, in a way that is complementary to the activity of Aurora B at the centromere (Ye *et al*., 2015). By interacting with Aurora A VirD5 might therefore still interfere with chromosome segregation in human cells as in yeast cells.

We found indeed chromosome mis-segregation in non-induced U2OS-TR cells transfected with the VirD5 construct at leaky low level expression of VirD5 (data not shown) and also lagging chromosomes after induction of VirD5 (**Figure 6**). Otherwise, chromosome pulverization, permanent cell cycle arrest and apoptosis were later seen in cells highly expressing VirD5 after induction with doxycycline (**Figure 8** and **9**). We observed DNA damage foci (γ-H2AX) in cells in the presence of VirD5 (**Figure 7**). How and when VirD5 induces DNA damage is still unknown. We have seen that VirD5 colocalizes with PCNA (**Figure 4**) in interphase cells. This may indicate that VirD5 interferes with DNA replication or that VirD5 causes DNA damage, which needs DNA replication for repair. Extensive DNA damage may explain the formation of broken chromosomes and micronuclei and our finding that VirD5 at higher concentrations leads to permanent arrest or apoptosis (**Figure 9**).

The effects of VirD5 seen at low level due to leaky expression under non-induced conditions may in fact better represent its natural biological effects and role in host cells during *Agrobacterium* infection, when it has to be delivered into the cells by the bacterial Type4 secretion system. *A. tumefaciens* naturally integrates the T-DNA into the host genome preferentially at double-stranded DNA break sites (Kim, Veena, and Gelvin, 2007; Salomon and Puchta, 1998). Limited DNA damage and break formation may therefore be advantageous for T-DNA integration. Also DNA damage may lead to temporary cell arrest by the DNA damage checkpoint allowing more time for T-DNA integration. A limited amount of chromosome mis-segregation in turn may favor the evolution of fast growing tumor cells promoting crown gall tumor formation.

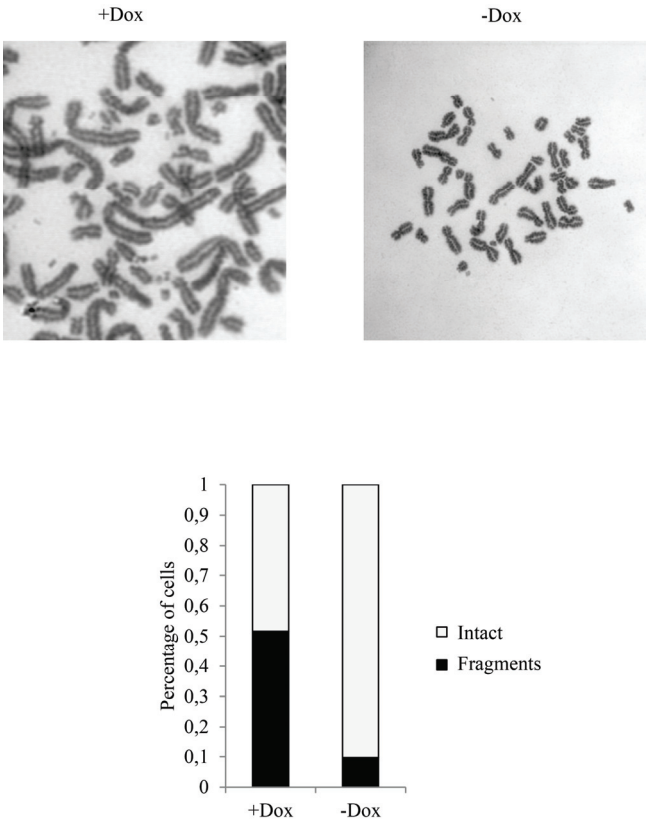

Figure 8. VirD5 triggers chromosome fragmentation. U2OS-TR-mCherry-VirD5 cells were treated with doxycycline or DMSO together with nocodazole. Chromosomes from metaphase cells were spread. 100 cells per experiment were observed.

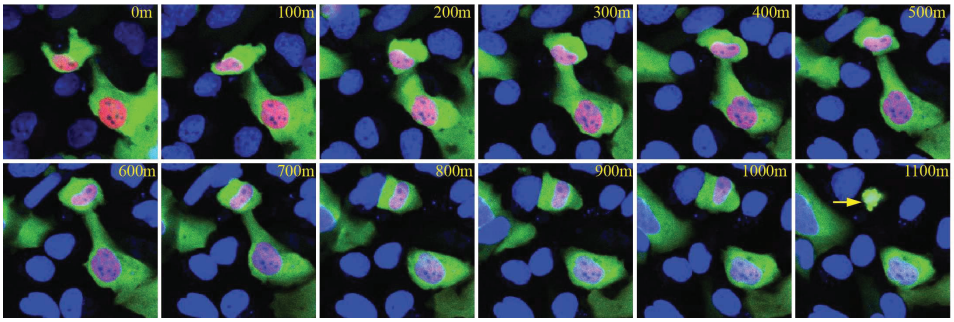

Figure 9. VirD5 induces apoptosis in human cells. Live-cell images of U2OS-TR cells transfected with plasmids encoding mCherry-VirD5 (red) and GFP-Tubulin (green) were taken after induction with doxycycline. Nuclei were stained by Hoechst. Images were taken every 4 minutes. Yellow arrow indicates the apoptotic cell.

Material and Methods

Cell culture

The human osteosarcoma cell line U2OS was maintained in DMEM (D1145, Sigma) containing 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin. A tetracycline inducible U2OS-TR cell line (a gift from Prof. Marc Timmers, UMC Utrecht) was grown in DMEM supplemented with 10% tetracycline-free FBS (631105, Clontech), 100 μg/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml hygromycin. Cells were grown at 37 ^{o}C in a humidified 5% CO_2 incubator.

Plasmids

Primers (**Table 1**)VirD5#43 or FlagF with either VirD5#23-2 or VirD5#40 were used to amplify the coding sequence of full-length VirD5 and the VirD5 N-terminal 202 amino acids part (VirD5N202) with or without the Flag tag. The VirD5 and VirD5N202 PCR products were digested with KpnI/SalI and KpnI/XhoI, respectively, and subcloned into KpnI/XhoI digested pCDNA3.1 (+) and pCDNA4/TO, generating pCDNA3.1 (+):VirD5/Flag-VirD5, pCDNA3.1 (+):VirD5N202/Flag-VirD5N202, pCDNA4/TO: VirD5/Flag-VirD5 and pCDNA4/TO: VirD5N202/Flag-VirD5N202. The mCherry fragment obtained from pCDNA3.1 (+):mCherry:apoptin after digestion with KpnI was inserted in frame into the above four plasmids lacking the Flag tag, yielding pCDNA3.1 (+):mCherry:VirD5, pCDNA3.1 (+):mCherry:VirD5N202, pCDNA4/TO: mCherry:VirD5 and pCDNA4/TO: mCherry:VirD5N202. The *AurkA, AurkB* and *AurkC* genes were amplified from pTomo-AurA, pDONR223-AURKB and pDONR223-AURKC (Addgene), respectively, using primers AurAF/AurAR, AurBF/AurBR, AurCF/AurCR, respectively. PCR fragments cut with SpeI and SalI were ligated into the XbaI/SalI fragment of pGEX-KG (Invitrogen) containing a GST tag and the SpeI/SalI fragment of BIFC vector pUG35VNC, resulting in pGEX-KG:AurKA, pGEX-KG:AurKB, pGEX-KG:AurKC, and pUG35VNc:AurKA, pUG35VNc:AurKB, pUG35VNc:AurKC, respectively. AurkA, AurkB, AurkC, GFP:VirD5 and Turquoise: VirD5 fragments were amplified using primer pairs AurkAmycF/AurAR, AurkBmycF/AurBR, AurkCmycF/AurCR, GFPfw2/VirD5#23-2 and TurquoiseF /VirD5#23-2, respectively. PCR products cut with KpnI/SalI were subcloned into KpnI/SalI digested pCDNA4/TO, forming pCDNA4/TO: AurkA, pCDNA4/TO: AurkB, pCDNA4/TO: AurkC, pCDNA4/TO:GFP:VirD5 and pCDNA4/TO: Turquoise:VirD5, respectively. All plasmids (**Table 2**) were sequenced by Sanger sequencing.

Indirect immunofluorescence assay

For most experiments, cells were seeded on glass coverslips, washed 3 times in PBS buffer and fixed in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 4% paraformaldehyde and 0.1% glutaraldehyde for 10 minutes at room temperature. After fixation, cells were quenched twice in freshly prepared 2mg/ml $NaBH_4$ for 10 minutes, followed by permeablization in PHEM containing 0.5% TritonX-100 for 10 minutes. hereafter, cells were blocked in TBST (TBS with 0.1% Tween20) with 5% BSA for 1 hour at room temperature and were incubated with primary antibody in TBST-BSA over night at 4 ^{o}C. Next, cells were washed 4 times in TBST buffer and incubated with fluorescence-conjugated secondary antibodies (Abcam) for 1 hour at room temperature. After

4 times washing in TBST, cells were stained for DNA with 0.2 μg/ml 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI:Sigma-Aldrich). Coverslips were mounted using a PolyMount Mounting Medium (Tebu-Bio, Heerhugowaard, Netherlands). The Primary antibodies used in this study were mouse anti-mCherry (1:500, Abcam), mouse anti-Hec1 (1:200, Abcam), rat anti-tubulin (YL 1/2, 1:500, Novus), anti-γ-tubulin (1:500, Sigma), rabbit anti-γ-H2AX (1:1000, Abcam), mouse-anti-Flag (1:100, Sigma),

Chromosome spread and karyotyping

U2OS-TR cells were transfected with pCDNA4/TO-mCherry-VirD5 via the polyethylenimine (PEI) transfection method. The stable cell line obtained was washed twice with PBS and re-cultured in fresh medium supplemented with 0.1 μg/ml nocodazole (Sigma) and 1 μg/ml Doxycycline or DMSO for 16 hours, followed by twice washing with warm PBS. Cells were trypsinized for 5 minutes and re-suspended in fresh medium. After 3 minutes centrifugation at 1200 rpm, cells were re-suspended with PBS and pelleted at 2000 rpm for 2 minutes. The pellet was re-suspended in a hypotonic solution of 0.56% KCl for 15 minutes at 37 ^{o}C. After that, cells were pelleted and fixed in cold Carnoy's fixative (3:1 methanol: glacial acetic acid) for 10 minutes on ice. Samples were re-suspended in fresh cold Carnoy's fixative. All fixed samples were spread on slides by dropping from a height of approximately 20 cm and shortly heat dried. Samples were stained with Giemsa for 30 minutes and rinsed with H_2O. Karyotyping pictures were imaged on the Zeiss Imager confocal microscope using a 63xoil objective.

In vitro Pull-down assay

GST, GST-AurKA, GST-AurkB and GST-AurkC were expressed in *E.coli* strain BL21DE3PLySs. Equal amounts of the GST-tagged proteins were immobilized on Glutathione HiCap Matrix (Qiagen, 30900) for 2 hours at room temperature. The beads with bound GST-tagged proteins-bound beads washed 3 times with PBS-EW buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 1 mM DTT, 1 mM EDTA), and then incubated with the purified His-tagged VirD5 protein in binding buffer (50 mM NaH_2PO4, 150 mM NaCl, pH 7.2, 0.1% Triton X-100) for additional 2 hours at room temperature. After 3 times washing with buffer (50mM Tris pH 8.0, 200 mM NaCl, 1 mM DTT, 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40), 20 μL sample buffer was mixed with samples for boiling, followed by centrifugation for 2 minutes at 2000 rpm. The supernatants were loaded to a 10% SDS-PAGE gel for electrophoresis. The presence of the His-tagged VirD5 proteins was detected with Anti-His HRP antibody (Santa Cruz Biotechnology, sc-8036 HRP) by Western Blot analysis.

BIFC

The pUG34VCn:VirD5 vector was cotransformed either with pUG35VNc:AurKA, pUG35VNc:AurKB or pUG35VNc:AurKC into wild type yeast cells (BY4743). The transformants were grown at 30 ^{o}C on MY media containing methionine to inhibit the expression of VirD5. After 3 days, colonies were picked to MY liquid media containing methionine. Overnight cultures were centrifuged and washed twice with sterilized water and then transferred into new flasks containing MY media lacking methionine to induce the expression of VirD5 for an additional 1 hour. Cells were harvested for BIFC signal

visualization using a 63xoil objective on the Zeiss Imager confocal microscope. Images were processed with ImageJ (ImageJ National Institutes of Health).

Co-immunoprecipitation

Detection of an *in vivo* interaction of the VirD5 protein with AurkB in U2OS-TR cells was carried out as follows. Either the mCherry-tagged VirD5 or mCherry-VirD5N202 plasmid was transfected with the vector containing the myc-tagged AurkB construct into U2OS-TR cells and incubated for 24 hours. Cells were washed twice with PBS and recultured for additional 24 hours in fresh medium containing 1μg/ml doxycycline to induce the expression of proteins. The induced cells were washed twice with cold PBS and lysed in 1x lysis buffer (Cell Signaling) with the addition of protease inhibitor (Roche) and 1mM PMSF, followed by incubation for 5 minutes on ice. The lysate was scraped and sonicated briefly; after that, the supernatant was obtained by centrifugation for 10 minutes at 4 ^{o}C at maximum speed. The myc-tagged AurkB protein and its binding protein were captured to the anti-myc antibody immobilized agarose (Pierce, 23620) according to the manufacturer's protocol; after 4 times washing with 1x lysis buffer, proteins were detected by Western blotting with anti-myc (Sigma) or anti-mCherry antibody (Abcam).

Long time live-cell imaging

U2OS-TR cells co-transfected with constructs expressing mCherry-VirD5 and GFP-Tubulin were grown on glass-bottom petri dishes (Willco Wells BV). 24 hours after transfection; then 1 μg/ml doxycycline was added to induce the expression of VirD5 and cells were grown for an additional 6 hours. Cells were imaged in a humid 37 ^{o}C chamber with 5% CO_2 using the Nikon EclipseTE2000-E microscope. Images were taken every 4 minutes.

Acknowledgements

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Table 1. Primers used in this study.

Primer	Sequence	Restriction site (underlined)
AurAF	GGACTAGTCATGGACCGATCTAAAGAAAAC	SpeI
AurAR	ACGCGTCGACGAGACTGTTTGCTAGCTGATTC	SalI
AurBF	GGACTAGTCATGGCCCAGAAGGAGAACTCC	SpeI
AurBR	ACGCGTCGACGGGCGACAGATTGAAGGGCAG	SalI
AurCF	GGACTAGTCATGAGCTCCCCCAGAGCTGTGG	SpeI
AurCR	ACGCGTCGACGGGAAGCCATCTGAGCACAG	SalI
AurkAmycF	GGGGTACCATGGAACAAAAACTTATTTCTGAAGAAGATCT GGACCGATCTAAAGAAAACTGC	KpnI
AurkBmycF	GGGGTACCATGGAACAAAAACTTATTTCTGAAGAAGATCT GGCCCAGAAGGAGAACTCCTAC	KpnI
AurkCmycF	GGGGTACCATGGAACAAAAACTTATTTCTGAAGAAGATCT GAGCTCCCCCAGAGCTGTGGTGC	KpnI
FlagF	GGGGTACCATGGACTACAAGGACGACGACGATAAG	KpnI
TurquoiseF	GGGGTACCATGGTGAGCAAGGGCGA	KpnI
VirD5#23-2	ACGCGTCGAC TCAGCGTTTAAAC	SalI
VirD5#40	CCGCTCGAGTCAACCATATGCAGAAC	XhoI
VirD5#43	GGGGTACCACAGGAAAGTCGAAAGTTC	KpnI

Table 2. Plasmids used in this study.

Plasmid name	Description
pCDNA3-mCherry-VirD5	mCherry-VirD5 (KpnI/SalI) was inserted into pCDNA3.1+
pCDNA3-mCherry-VirD5N202	mCherry-VirD5N202 (KpnI/XhoI) was inserted into pCDNA3.1+
pCDNA3-Flag-VirD5	Flag-VirD5 (KpnI/SalI) was inserted into pCDNA3.1+
pCDNA3-Flag-VirD5N202	Flag-VirD5N202 (KpnI/XhoI) was inserted into pCDNA3.1+
pCDNA3-GFP-PCNA	A gift from M. Cristina Cardoso
pCDNA4/TO-mCherry-VirD5	mCherry-VirD5 (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-mCherry-VirD5N202	mCherry-VirD5N202 (KpnI/XhoI) was inserted into pCDNA4/TO
pCDNA4/TO-Flag-VirD5	Flag-VirD5 (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-Flag-VirD5N202	Flag-VirD5N202 (KpnI/XhoI) was inserted into pCDNA4/TO
pCDNA4/TO-GFP-VirD5	GFP-VirD5 (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-Turquoise-VirD5	Turquoise-VirD5 (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-myc-AurkA	myc-AurkA (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-myc-AurkB	myc-AurkB (KpnI/SalI) was inserted into pCDNA4/TO
pCDNA4/TO-myc-AurkC	myc-AurkC (KpnI/SalI) was inserted into pCDNA4/TO
pET16H-VirD5	VirD5 (XmaI/ClaI) was inserted into pET16H
pGEX-KG-AurkA	AurkA (SpeI/SalI) was inserted into pGEX-KG
pGEX-KG-AurkB	AurkB (SpeI/SalI) was inserted into pGEX-KG
pGEX-KG-AurkC	AurkC (SpeI/SalI) was inserted into pGEX-KG
pGEX-KG-Ipl1-NT (1-118)	Ipl1-NT (1-118) (XmaI/SalI) was inserted into pGEX-KG
pGEX-KG-Ipl1-CT (101-367)	Ipl1-CT (101-367) (XmaI/SalI) was inserted into pGEX-KG
pUG34VCN	(Sakalis, 2013)
pUG34VCN-VirD5	VirD5 (SpeI/SalI) was inserted into pUG34VCN
pUG35VNC	(Sakalis, 2013)
pUG35VNC-AurkA	AurkA (SpeI/SalI) was inserted into pUG35VNC
pUG35VNC-AurkB	AurkB (SpeI/SalI) was inserted into pUG35VNC
pUG35VNC-AurkC	AurkC (SpeI/SalI) was inserted into pUG35VNC

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Summary and discussion

The Gram-negative soil bacterium *Agrobacterium tumefaciens* is capable of infecting a large number of dicotyledonous plants, causing crown gall disease. During infection, a small DNA segment (T-DNA) from the tumor-inducing (Ti) plasmid of the bacterium is delivered via a VirB/D4 Type Four Secretion System (TFSS) into plant cells where it is integrated into the genome (Christie, 2004). The expression of genes on the T-DNA in transformed plant cells leads to uncontrolled cell division, and ultimately triggers plant tumor formation. *Agrobacterium* also transfers several virulence (vir) proteins (VirD2, VirD3, VirD5, VirE2, VirE3 and VirF) encoded by genes present in the vir region of the Ti plasmid to host cells via the same TFSS apparatus independently of the T-DNA (Vergunst *et al*., 2000). In this study, I mainly focused on the biological functions of one of these translocated virulence proteins called VirD5.

It has been shown that budding yeast is an excellent model organism for studies of the biological functions of bacterial virulence proteins. We thus expressed the VirD5 proteins from different *Agrobacterium* strains in yeast cells in **Chapter 2** and found that they all inhibited the growth of yeast. The VirD5 protein was fused to the GFP protein and thus it was found that VirD5 was present in the nucleus, where it was present at a small number of foci, representing the centromeres/kinetochores. From a genome-scale deletion mutant library screening we found that the toxicity of VirD5 could be suppressed by nine deletion mutations. In one of these suppressive deletion mutants the *SPT4* gene was absent, which is involved in kinetochore assembly and transcriptional elongation (Crotti and Basrai, 2004). In the absence of the Spt4 protein VirD5 was no longer targeted to the centromeres/kinetochores of yeast cells. Further molecular experiments showed that VirD5 physically interacted with the Spt4 protein, indicating that Spt4 might assist in the accumulation of VirD5 at the centromeres/kinetochores. The interaction with Spt4 and the sublocation at the centromeres/kinetochores are mainly attributed to the N-terminal part of VirD5 (VirD5NT), overexpression of which also displayed growth inhibition. Targeting to the centromeres/kinetochores led to chromosome mis-segregation, aneuploidy and DNA damage, all hallmarks of cancer cells.

In **Chapter 3,** we did further experiments in order to better understand the molecular mechanism underlying the toxicity of VirD5, We found that VirD5 specifically binds to centromeric DNA *in vitro*, but this DNA binding activity was not strong enough to bring VirD5 to the centromeres/kinetochores *in vivo*. The kinetochore is a large protein complex consisting of more than 60 proteins in yeast cells, which is assembled on each of the centromeres (Biggins *et al*., 1999; Yamagishi *et al*., 2014). Therefore, we surveyed among the kinetochore proteins for potential interaction partners of VirD5 using the Bimolecular Fluorescent Complementation (BIFC) assay, and found that the microtubule-embracing protein Dam1 and the mitotic regulatory Ipl1/Aurora kinase displayed a strong interaction signal with VirD5 and VirD5NT (1-505). The interaction with VirD5 stimulated the kinase activity of Ipl1/Aurora kinase *in vitro*, which could lead to chromosome mis-segregation *in vivo*. That activation of the Ipl1/Aurora kinase underlies the toxicity of VirD5NT became

apparent by boosting of the activity of the specific counteracting phosphatase Glc7 (Pinsky *et al*., 2006), which relieved the toxicity of VirD5 *in vivo*.

In **Chapter 4** it was shown that the VirD5 protein causes chromosome mis-segregation and aneuploidy in plant cells and that it may do so by targeting the three plant mitosis regulatory Aurora kinases, namely Aurora 1, Aurora 2 and Aurora 3. An *Agrobacterium* strain lacking VirD5 induced plant tumors of similar size as the wild type strain containing VirD5. However, an *Agrobacterium* strain harboring a *virD5* gene driven by plant transcriptional regulatory sequences in the T-DNA of a binary vector only induced extremely small tumors, suggesting that the high dose of VirD5 negatively affected division of plant tumor cells.

Chapter 5 disclosed the physical interaction of VirD5 exclusively with the C-terminal catalytic domain of the Ipl1/Aurora kinase. This C-terminus is conserved between Aurora kinases from different organisms. We thus examined whether VirD5 had also toxic effects in human cells. First of all we found that VirD5 interacted with the three mammalian Aurora kinases, AurkA, AurkB and AurkC which are essential for accurate chromosome segregation in mammalian cells during mitosis (Fu *et al*., 2007). In human cells, VirD5 was localized at DNA replication sites in interphase cells, but it accumulated at the centrosomes in mitotic cells. Ectopic expression of VirD5 in mammalian cells caused chromosome mis-segregation, DNA damage, micronuclei formation and apoptosis.

As a conclusion this study illustrates that the virulence protein VirD5, which is translocated by *Agrobacterium tumefaciens* into eukaryotic cells, targets the essential mitotic regulatory Aurora kinases of the host cells. Targeting of the Aurora kinases by VirD5 may stimulate their kinase activities on key substrates involved in mitosis and consequently arrest the cell cycle, causing DNA damage, chromosome mis-segregation and aneuploidy, all hallmarks of cancer cells. During infection, *Agrobacterium tumefaciens* transfers and integrates the T-DNA into host cells at DNA breaks. We thus propose that the DNA breaks caused by VirD5 may lead to mutation and offer potential insertion site for T-DNA. The invoked cell cycle arrest might also be beneficial for the T-DNA integration. Besides the resulting mutations and aneuploidy may generate the fast growing tumor cells that grow uncontrolled and in the end form the majority of the tumor.

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Samenvatting en discussie

De Gram-negatieve bacterie Agrobacterium tumefaciens kan een groot aantal tweezaadlobbige planten infecteren, met als gevolg de vorming van wortelhalsknobbels ("kroongal ziekte"). Tijdens de infectie wordt een klein stukje DNA (T-DNA genaamd) door de bacterie overgebracht in plantencellen. De expressie van genen op het T-DNA in de getransformeerde plantencellen leidt tot ongecontroleerde celdeling en uiteindelijk tot tumorvorming op de geinfecteerde planten. Het T-DNA maakt in de bacterie onderdeel uit van het tumor inducerende (Ti) plasmide van de bacterie. Op dit Ti plasmide liggen ook de genen, die het VirB / D4 Type4 secretie systeem (TFSS) coderen dat verantwoordelijk is voor de translocatie van het T-DNA in plantencellen. Agrobacterium brengt via dit TFSS ook verschillende virulentie (vir) eiwitten (VirD2, VirD3, VirD5, VirE2, VirE3 en VirF) over in plantencellen tijdens de infectie onafhankelijk van het T-DNA transport (Vergunst *et al*., 2000). In het promotieonderzoek heb ik mij vooral gericht op de biologische functies van één van deze getransloceerde virulentie eiwitten genaamd VirD5.

Toen bleek dat VirD5 toxisch was voor plantencellen en het daarom lastig was de functie van VirD5 te bestuderen in plantencellen, ben ik overgestapt op bakkersgist, dat ook getransformeerd kan worden door Agrobacterium (Bundock *et al*., 2005). In eerdere studies was namelijk gebleken dat gist een uitstekend modelorganisme is voor studies van de biologische functies van bacteriële virulentie eiwitten. Allereerst vonden we dat de VirD5 eiwitten van verschillende Agrobacterium stammen, die licht van elkaar verschilden, ook de groei van gistcellen remden (**hoofdstuk 2**). Het VirD5 eiwit werd vervolgens gefuseerd aan het GFP-eiwit en aan de hand van fluorescentie kon worden vastgesteld, dat VirD5 aanwezig was in de kern van de gistcellen. In de kern was VirD5 geconcentreerd op een klein aantal spots, overlappend met de centromeren / kinetochoren. Via de screening van een genoom-brede deletie mutant bibliotheek vonden we dat de toxiciteit van VirD5 kon worden onderdrukt door negen deletie mutaties. In één van deze onderdrukkende deletiemutanten was het SPT4 gen afwezig, dat onder andere betrokken is bij kinetochoor assemblage (Crotti en Basrai , 2004). In afwezigheid van het Spt4 eiwit kon VirD5 niet meer binden aan de centromeren / kinetochoren in gistcellen. In verdere experimenten kon worden aangetoond dat VirD5 een directe fysische interactie aangaat met het Spt4 eiwit, hetgeen indiceert dat Spt4 verantwoordelijk is voor de accumulatie van VirD5 op centromeren / kinetochoren. Het N-terminale deel van VirD5 (VirD5NT) is betrokken bij de interactie met Spt4 en is nodig voor de localisatie van VirD5 op de centromeren / kinetochoren en daarmee verantwoordelijk voor de remming van de groei. Door de aanwezigheid op de centromeren / kinetochoren veroorzaakt VirD5 chromosoom mis-segregatie, aneuploïdie en DNA-schade, alle kenmerken van kankercellen.

In **hoofdstuk 3** zijn verdere experimenten gedaan om beter inzicht te verkrijgen in de moleculaire mechanismen, die ten grondslag liggen aan de toxiciteit van VirD5. We vonden dat VirD5 specifiek bindt aan het DNA van de centromeren *in vitro*, maar deze DNA-bindende activiteit was niet sterk genoeg om VirD5 op de centromeren / kinetochoren te localiseren *in vivo*. Het kinetochoor is een groot eiwitcomplex, dat is gebonden aan elk van de centromeren en bestaat uit meer dan 60 verschillende eiwitten in gistcellen (Biggins *et al*,

1999;. Yamagishi *et al*, 2014). Daarom hebben we vervolgens gezocht onder de kinetochoor eiwitten voor mogelijke interactie partners van VirD5 met behulp van de Bimoleculaire Fluorescentie Complementatie (BiFC) test. We vonden met deze test dat zowel het eiwit Dam1, dat ringen vormt rond de uiteinden van de microtubuli, als het Ipl1 / Aurora kinase, een essentiele regulator van mitose, een sterke interactie aangaat met VirD5 en VirD5NT (1-505). De interactie met VirD5 stimuleerde de kinase activiteit van Ipl1 / Aurora kinase *in vitro*, hetgeen ten grondslag zou kunnen liggen kunnen aan de onjuiste chromosoom segregatie *in vivo*. Dat activatie van de Ipl1 / Aurora kinase inderdaad ten grondslag ligt aan de toxiciteit van VirD5NT werd duidelijk, toen bleek, dat de toxiciteit van VirD5 *in vivo* kon worden opgeheven door het stimuleren van de activiteit van het specifieke tegenwerkende fosfatase Glc7 (Pinsky *et al*., 2006).

In **hoofdstuk 4** werd aangetoond dat het VirD5 eiwit bindt aan de drie centrale mitose regulerende Aurora kinasen van planten, Aurora 1, Aurora 2 en Aurora 3 en ook in plantencellen chromosoom mis-segregatie en aneuploïdie veroorzaakt. Een Agrobacterium stam, waarin VirD5 ontbreekt, induceerde plantentumoren van vergelijkbare grootte als de wild-type stam met VirD5. Blijkbaar is VirD5 niet essentieel voor de initiatie van tumorvorming. Wanneer tijdens de infectie in de geinfecteerde plantencellen echter een hogere dosis VirD5 accumuleerde door expressie van een *virD5* gen, dat was gecloneerd in het T-DNA van een binaire vector en dat onder controle was gebracht van de transcriptionele regulerende sequenties van een plantengen, werden slechts zeer kleine tumoren geïnduceerd, hetgeen liet zien dat een hogere dosis VirD5 deling van plantaardige tumorcellen negatief beïnvloedt.

In **hoofdstuk 5** werd beschreven dat VirD5 uitsluitend interacteert met het C-terminale katalytische domein van Ipl1 / Aurora kinase. Deze C-terminus is sterk geconserveerd in de Aurora kinasen van verschillende organismen. Daarom werd onderzocht of VirD5 niet alleen toxisch was voor gistcellen en plantencellen, maar ook voor humane cellen. Er werd gevonden dat VirD5 inderdaad ook interacteert met de drie zoogdier Aurora kinasen, AurkA, AurkB en AurkC die essentieel zijn voor accurate chromosoomsegregatie in zoogdiercellen tijdens mitose (Fu *et al*., 2007). In humane cellen was VirD5 tijdens de interfase gelokaliseerd op mogelijke DNA replicatie plaatsen in de kern, maar het was tijdens de mitose met name aanwezig op de centrosomen. Ectopische expressie van VirD5 in zoogdiercellen veroorzaakte chromosoom mis-segregatie, DNA-schade, de vorming van micronuclei en apoptose.

Samenvattend kan worden geconcludeerd dat het virulentie eiwit VirD5, dat door *Agrobacterium tumefaciens* in eukaryote cellen wordt getransloceerd, de pijlen richt op de voor mitose essentiële regulerende Aurora kinasen van de gastheercellen. Stimulering van de kinase activiteit van de Aurora kinasen door VirD5 kan leiden tot verhoogde fosforylering van bepaalde substraten, die een belangrijke rol spelen bij de mitose en bijgevolg tot een vertraging van de celcyclus en in de anafase achterblijvende chromosomen. Hiervan kan DNA-schade, verkeerde chromosoom segregatie, vorming van micronuclei en aneuploïdie het gevolg zijn, alle kenmerken van kankercellen. Voor een succesvolle infectie, moet het T-DNA van *Agrobacterium tumefaciens* geintegreerd worden in het genoom van de gastheercellen. De DNA-breuken die worden veroorzaakt door aanwezigheid van VirD5 bieden mogelijk een insertieplaats voor het T-DNA. Vertraging van de celcyclus kan ook

gunstig zijn voor de integratie van het T-DNA. Echter een te hoge dosis VirD5 kan leiden tot een te grote DNA beschadiging en celdood, en dus tot gereduceerde tumorvorming. Bij lage dosis zou de door VirD5 veroorzaakte DNA schade kunnen leiden tot mutatie en de effecten van VirD5 op chromosoom segregatie tot aneuploidie. Mutatie en aneuploïdie zijn ook kenmerkend voor humane tumorcellen en zouden aanleiding kunnen zijn tot de vorming en selectie van de snel groeiende tumorcellen die uiteindelijk de meerderheid van het plantentumorweefsel genereren.

Curriculum vitae

Xiaorong Zhang was born on the 6th of November 1986 in Huai'an, Jiangsu Province, China. In 2003 he started his studies at Soochow University and obtained his Bachelor diploma in Bioinformatics in 2007. He continued his Master studies in Molecular Genetics at Soochow University under the supervision of Prof. Guangli Cao and Prof. Dr. Chengliang Gong, and received his Master degree in 2010. In October 2010, he started his PhD project which concerns functional analysis of *Agrobacterium tumefaciens* virulence protein VirD5 under the supervision of Prof. Dr. Paul J.J. Hooykaas at the Institute of Biology, Leiden University, the Netherlands, with a financial support by China Scholarship Council. In September 2014, he continued to work as a postdoc to elucidate the biological role of VirD5 under the supervision of Prof. Dr. Paul J.J. Hooykaas.